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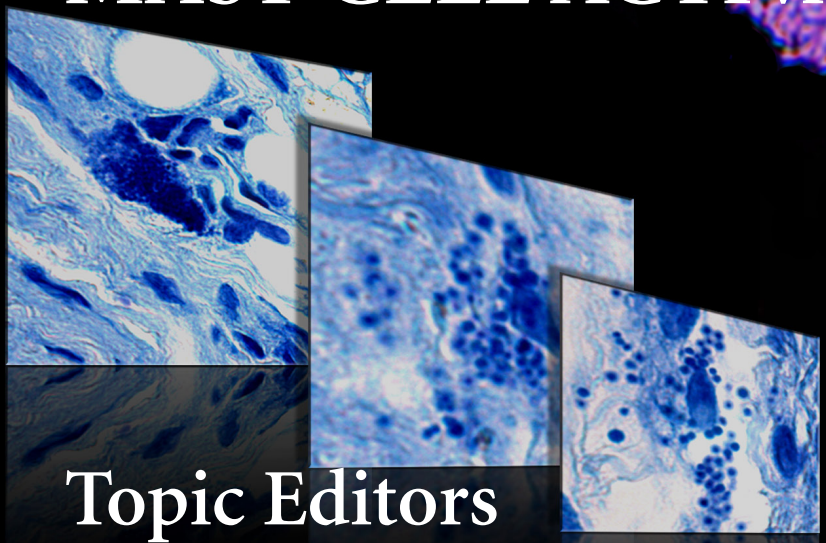
DECIPHERING NEW MOLECULAR MECHANISMS OF MAST CELL ACTIVATION



Topic Editors
Ulrich Blank and Marc Benhamou

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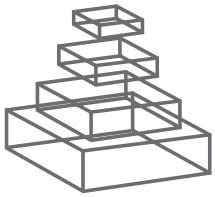

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DECIPHERING NEW MOLECULAR MECHANISMS OF MAST CELL ACTIVATION

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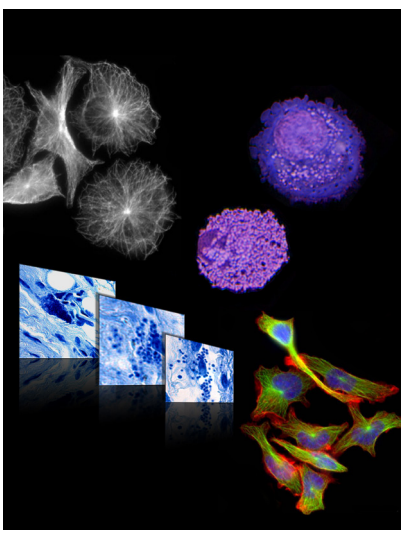

Mast cells are tissue-localized cells that play an important role in immunity and inflammation. Following an offensive event they act as cellular sensors that via the activation of cell surface receptors launch a cellular response culminating in the release of a whole set of inflammatory mediators and products. This response is initially destined to restore tissue homeostasis, but in case of chronic injury or deregulation also promotes pathology. To further understand the action of mast cells in their environmental context it is necessary to decipher the molecular mechanisms of their activation as well as the ensuing cellular responses. This will allow identification of new strategies to promote their beneficial actions or, at the contrary, to interfere with their pathological consequences. While in the past many studies have focused on responses engaged by high affinity IgE receptor because of its implication in the allergic response, it has become clear that mast cells can be activated by multiple types of receptors initiating an intense molecular crosstalk between receptors and signaling pathways that can either synergize, antagonize and in some cases produce new types of responses. Mast cells can indeed react with an astounding diverse array of cellular responses that sometimes are engaged selectively. This "Research Topic" will focus on selected articles that shed some new light on the molecular mechanisms of mast cell activation, the possible crosstalk between signaling pathways and the ensuing cellular responses that allow mast cells to act as cellular sensors in tissues.

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EDITORIAL
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Deciphering new molecular mechanisms of mast cell activation

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Mast cell were initially studied mostly for their implication in type I hypersensitivity and allergies (Blank and Rivera, 2004; Galli et al., 2008b). However, research over the last 20 years has made clear that they do more than just causing a runny nose or asthma attacks. In fact, they have emerged as prime actors of immune and inflammatory responses. As such they sense their environment via numerous cell surface expressed receptors to orchestrate an appropriate immune and inflammatory response. Work from many laboratories has made clear that mast cells participate in the restoration of tissue homeostasis following an offensive event contributing for example to the elimination of infectious agents as well as to tissue repair and remodeling responses (Marshall, 2004; Galli et al., 2005; Blank et al., 2007; Abraham and St John, 2010; Beghdadi et al., 2011).

Mast cells are tissue-localized effectors of hematopoietic origin. They are particularly prominent in tissues that are in contact with the external environment such as the skin, the gastrointestinal tract, or the airways. Yet, they are present also in many other tissues of an organism and often their numbers increase upon an inflammatory reaction. They can be found at strategic locations close to blood capillaries in order to rapidly communicate to other hematopoietic effectors to enter into action. They are also found in contact with nerve terminals allowing these cells, via released mediators, to communicate to the brain the presence of environmental dangers or reversely to respond to signals emanating from the brain, thus providing a relay between the central nervous system and the immune system (Theoharides et al., 2012).

One of the prime characteristics of mast cells is to release rapidly within a few minutes upon activation a whole set of inflammatory products by cellular degranulation (**Figure 1**) including histamine and proteases, proteoglycans, lysosomal enzymes, etc. (Blank and Rivera, 2004). As a consequence, blood vessels dilate thus increasing the blood flow in the offended area. Moreover, under the effect of released histamine, vessels become permeable allowing the influx of other inflammatory cells and other inflammatory products (such as for example immunoglobulins and complement) into tissues to mount an inflammatory response against the insult. In a more delayed response mast cells also produce lipid mediators such as prostaglandins and leukotrienes, which enhance some of the initial tissue responses and promote supplementary responses such as increased temperature (fever), smooth muscle contraction, etc. This is followed by a third wave of mediators, which correspond to various cytokines and chemokines allowing to enhance the flow of other inflammatory and immune cells into tissue, but also to start to regulate and coordinate this inflammatory response. Interestingly, the array of mediators released may differ depending on the type of stimulus. Thus, stimulation of Toll-like receptor 4 (TLR4) by bacterial products such as LPS usually does not promote the first wave of degranulation, but rather leads to an enhanced production of cytokines and chemokines (Leal-Berumen et al., 1994). Therefore, in addition to releasing a whole set of inflammatory mediators, mast cells can initiate different actions allowing these cells to fine-tune the inflammatory response. Indeed, evidence has emerged that mast cells, depending on the type of stimulus initiate different actions that can have opposite pro- or anti-inflammatory consequences (Metz et al., 2007; Galli et al., 2008a; Beghdadi et al., 2011). Interestingly, in some cases this may depend on the strength of the stimulus or on its timing in the course of an inflammatory reaction (Beghdadi et al., 2011).

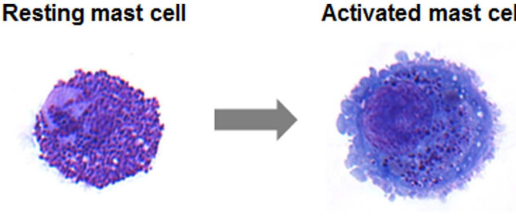

FIGURE 1 | May–Grünwald/Giemsa stain of a resting human intestinal mast cell and a mast cell following activation induced degranulation. Note the increase in size and loss of granule staining (Figure from Lorentz et al., 2012).

While in the past many reviews have focused on the capacity of these cells to respond to IgE-mediated activation, the focus of this ebook of *Frontiers in Innate Immunology* is devoted substantially to emphasize other types of stimuli for mast cells and the molecular mechanisms involved, as well as to highlight new types of responses by these cells. Thus, several reviews of this ebook describe new types of stimuli for these cells involving chemokines (Halova et al., 2012), sex hormones (Zierau et al., 2012), tetraspanins (Koberle et al., 2012), TRP channels (Freichel et al., 2012), TLRs (Sandig and Bulfone-Paus, 2012). Another article provides a general description of novel identified receptors (Migalovich-Sheikhet et al., 2012) that may act also as inhibitory receptors of an inflammatory reaction. Some contributions deal with the regulation of mast cell activation involving for example cytoskeletal elements (Draber et al., 2012), ion channels or tetraspanins (Freichel et al., 2012; Koberle et al., 2012), or the mechanism

involved in secretory granule fusion or the crosstalk between different cell surface receptors (Lorentz et al., 2012; Migalovich-Sheikhet et al., 2012). Another point discussed is the implication of these stimuli in new types of biological responses mediated by mast cells. This includes for example a description of how female sex hormones can influence allergic asthma, how these hormones participate in mast cell uterine functions (Zierau et al., 2012) and how mast cells can interfere with reproductive processes (Woidacki et al., 2013). Another chapter analyses the interaction of mast cells with other immune cells discussing the receptors and mediators involved in these new types of connections (Gri et al., 2012).

Thus, while in the past mast cells have been often discussed with respect to their participation in allergic type of responses this collection of specific chapters aims to stress that these cells are in fact versatile inflammatory effectors with multiple functions in the organism. Indeed, cells resembling mast cells that contain histamine and proteoglycans such as heparin have already been recognized in tunicates (Cavalcante et al., 2002), which are amongst the first multicellular organism preceding vertebrates. This is well before the appearance of IgE, making clear that these cells probably are part of an ancient immune surveillance system allowing the organism to defend itself against tissue damage and organize physiological responses within tissues.

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Tetraspanins in mast cells

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Mast cells (MC) are key mediators of the immune system, most prominently known for their role in eliciting harmful allergic reactions. Mast cell mediator release (e.g. by degranulation) is triggered by FcεRI recognition of antigen – IgE complexes. Until today no therapeutic targeting of this and other mast cell activation pathways is established. Among possible new candidates there are tetraspanins that have been described on MC already several years ago. Tetraspanins are transmembrane proteins acting as scaffolds, mediating local clustering of their interaction partners, and thus amplify their activities. More recently, tetraspanins were also found to exert intrinsic receptor functions. Tetraspanins have been found to be crucial components of fundamental biological processes like cell motility and adhesion. In immune cells, they not only boost the effectiveness of antigen presentation by clustering MHC molecules, they are also key players in all kinds of degranulation events and immune receptor clustering. This review focuses on the contribution of tetraspanins clustered with FcεRI or residing in granule membranes to classical MC functions but also undertakes an outlook on the possible contribution of tetraspanins to newly described mast cell functions and discusses possible targets for drug development.

Keywords: mast cell, tetraspanin, CD9, CD63, CD81, CD151

INTRODUCTION

Tetraspanins are evolutionary conserved and are expressed ubiquitously (Levy and Shoham, 2005). They are composed of the four eponymous transmembrane (TM) domains, connected by a small and a large extracellular as well as an intracellular loop (**Figure 1**). The large extracellular loop consists of two subdomains, a conserved three-helix structure that contains a canonical CCG motif and a variable region that is inserted within the conserved subdomain and is responsible for some of the specific protein-protein interactions of individual tetraspanins. A stretch of residues located in the TM2 – intracellular loop – TM3 region is also highly conserved and referred to as the tetraspanin signature. In the conserved regions, there are a number of canonical cysteine residues that ether serve as palmitoylation sites or form disulfide bonds that establish the topology of the large extracellular region (Seigneuret et al., 2001). Tetraspanins have a different primary sequence and do not share any homologies with the multigene four-transmembrane family proteins (MS4A) that comprise, e.g., CD20 and the high-affinity IgE receptor β chain (FcεRIβ), although the secondary structure of these proteins also forms four transmembrane domains (Ishibashi et al., 2001). Tetraspanins are able to form homodimers and to cluster with a large number of transmembrane proteins like integrins, major histocompatibility complex proteins and Fc receptors, but also with intracellular signaling molecules like phosphatidylinositol-4-kinase or RhoA. The clustering of tetraspanins leads to the formation of distinct membrane domains called tetraspanin enriched microdomains (TEM; **Figure 2**). By spatial enrichment of their binding partners, they bring together interacting elements of signaling pathways and also cluster homotypic signaling, thus amplifying signaling amplitudes (Hemler, 2005; Yanez-Mo et al., 2009).

Tetraspanin research started in the 1980s. While some were soon established as important cell surface markers, others were long neglected. Major problems were that no mouse antibodies could be raised against some human tetraspanins or that no biological functions could be elucidated because initially no classical receptor/ligand functions were observed (Hemler, 2005; Yanez-Mo et al., 2009).

In the last years, exciting new functions have been elucidated for tetraspanins. Not only their role as scaffold proteins is increasingly appreciated, e.g., in antigen presentation by dendritic cells (Unternaehrer et al., 2007), but also direct action of tetraspanins as receptors has been described lately, e.g., for IL-16 (Qi et al., 2006) or hepatitis C virus entry (Pileri et al., 1998).

Mast cells (MC) are tissue-homing immune cells that differentiate from blood borne committed progenitor cells and have been identified in all vertebrate classes (Crivellato and Ribatti, 2010). Preferentially populating surface organ tissues such as the skin or mucosal surfaces in the gut, MC contribute to the first line defense system against pathogens (Echtenacher et al., 1996). The discovery that MC are much more than an effector cell of type I allergic reactions challenged a paradigm, but was soon confirmed by other studies (for review see (Sayed et al., 2008; Abraham and St John, 2010; Beghdadi et al., 2011). In addition, MC were shown to be crucial mediators of delayed type hypersensitivity reactions (DTHR) of the skin (Biedermann et al., 2000), joints (Lee et al., 2002; Kneilling et al., 2007) and brain (Secor et al., 2000).

While some of these MC functions were questioned recently based on the model systems used (Zhou et al., 2007; Dudeck et al., 2011; Feyerabend et al., 2011), the critical role of MC for cutaneous DTHR was confirmed in different models (Biedermann

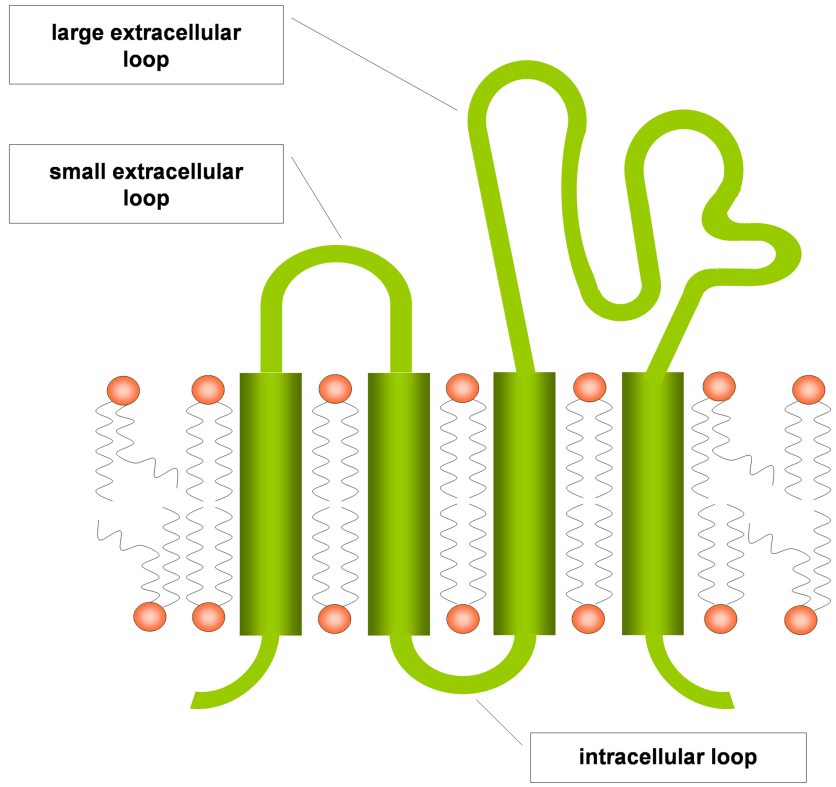

FIGURE 1 | Tetraspanin structure scheme (modified from Levy and Shoham, 2005).

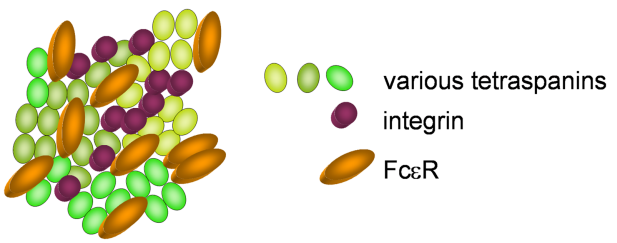

FIGURE 2 | Clustering of various tetraspanins, with integrins and Fcε receptors, forming a tetraspanin enriched microdomain.

et al., 2000; Dudeck et al., 2011; Otsuka et al., 2011). Moreover, it was discovered that MC functions are determined by the surrounding microenviroment of their destination and different situations of, e.g., inflammation or regeneration allow MC to adapt "as needed" (Pennock and Grencis, 2004, for review see Hallgren and Gurish, 2007; Collington et al., 2011). Signals and pathways involved in these adaptive processes have not been well characterized. In contrast, molecular pathways triggered by IgE-mediated MC activation have been studied in detail.

In mammals, MC express tetrameric high-affinity receptors for IgE (FcεRI) as well as stem cell factor receptors (c-kit, CD117) on the cell surface. Signaling events downstream of FcεRI are mediated by a large multi-protein complex, sometimes called the FcεRI signalosome mainly consisting of kinases and adaptor proteins (Draber et al., 2012). Upon FcεRI crosslinking by antigen – IgE complexes large intracellular granules are released that store a variety of mediators, among them histamine and proteases (tryptases, chymases; Amin, 2012). Additionally, upon activation they synthesize from arachidonic acid leukotrienes (e.g. leukotriene C_4) that add to the recruitment of leukocytes (Mandal et al., 2008). These features highlight MC as key players in allergic and inflammatory reactions. In addition, subsequently to degranulation after FcεRI crosslinking mediator release from MC is continued, contributing to delayed immune responses following MC activation. Some of the very early events following FcεRI crosslinking even distinguish between mediator release from granules and cytokine production including downstream immune consequences (Shumilina et al., 2008; Sobiesiak et al., 2009). In the last decade, MC and MC mediators have been increasingly appreciated as instructors of other cells in immune reactions by the release of cytokines or cell contact dependent signaling (Bradding et al., 1994; Sayed et al., 2008). For example, MC derived TNF has been shown to be crucial for neutrophil recruitment and local endothelial inflammation (Biedermann et al., 2000; Kneilling et al., 2009). While most prominently associated with allergic and autoimmune diseases as well as infections, MC and MC derived mediators also play an important role in the detoxification of insect or snake venoms (Metz et al., 2006; Akahoshi et al., 2011). This may be especially interesting, as it indicates also a physiologic relevance of venom specific IgE and IgE-dependent MC activation: low

level sensitization and IgE-mediated MC activation may have contributed to survival following intoxications and lead to a positive selection of this activation pathway during evolution.

By and large, tetraspanin expression and function in MC and basophils and in the variety of responses in which MC and basophils contribute have not been very well characterized. However, it is clear that tetraspanins play an important role in MC functions, because they are closely linked to FcεRI signaling and degranulation. Furthermore, they may also play an important role in some of the rather newly described MC functions, but this remains to be determined.

DISTRIBUTION AND FUNCTION OF TETRASPANINS IN MC AND BASOPHILS

CD9

On their search for an antibody specific to lymphohematopoietic progenitors, Kersey et al. (1981) identified CD9 (TSPAN29, Leukemia-Associated Cell Surface Antigen p24, Motility-Related Protein-1) by a monoclonal antibody (mAb) that bound to acute lymphoblastic leukemia cells.

CD9 is expressed on a wide variety of cells, including B cells, T cells, dendritic cells, platelets, and endothelial cells. By immunophenotyping human MC, CD9 has been detected on subtypes such as bone marrow MC, skin MC, renal MC, tonsillar MC, uterine MC, cardiac MC, lung MC as well as on basophils (Guo et al., 1992; Sperr et al., 1994; Fureder et al., 1997; Beil et al., 1998; Escribano et al., 1998; Ghannadan et al., 1998). Malignant MC clones express CD9 as well, though in contrast to CD63 no upregulation compared to healthy MC has been described (Baghestanian et al., 1996; Jost et al., 2001).

Data gained from experiments with platelets show co-localization and most likely functional interaction of CD9 and FcγRII, which is also the predominant FcγR in MC (Huang et al., 1992; Moseley, 2005). Degranulation of rat basophil leukemia (RBL) cells transfected with CD9 could be triggered by mAb directed against CD9 but not with $F(ab')_2$ fragments of these mAb. Therefore, these data argue that the artificial mAb treatment acts as a surrogate for IgE coated antigen leading to FcεRI clustering as well. While co-localization of CD9 and FcεRI was also proven by co-precipitation, no functional consequences were documented (Higginbottom et al., 2000).

IL-16 has been described as CD4 ligand inducing T helper cell chemotaxis and IL-2 receptor expression (Parada et al., 1998). However, MC migrate and maturate in response to IL-16 as well and while some MC lines and human cord blood derived MC do express CD4, human bone marrow and peripheral MC are CD4 negative. Even in the case of CD4 expression, IL-16 dependent migration is abrogated only partially by a CD4 blocking antibody treatment of MC and other monocytes (Sperr et al., 1994; Kirshenbaum et al., 2003; Qi et al., 2006). In the quest for an additional receptor, unexpectedly CD9 was identified as a receptor conferring IL-16 responsiveness to MC by induction of PI3K signaling and Ca^{2+} influx (Qi et al., 2006).

CD9, CD81 (see below), and FcεRI have been shown to be co-expressed and co-localized in human dendritic cells. In these cells, co-activation of FcεRI (by crosslinking) and CD9 (by mAb) resulted in increased IL-10 production (Peng et al., 2011).

A CD9 blocking antibody exists (Chen et al., 1999), and was shown to block MC migration *in vitro* (Qi et al., 2006). However, and in contrast to some other MC tetraspanins, it is unknown whether it affects degranulation. If so, it could be used in combination with other tetraspanin blocking antibodies and thus might synergistically increase the effectiveness of a tetraspanin targeting treatment option intended to ameliorate the symptoms of erratic MC activation in allergic disease. If it furthermore blocks chemotaxis also *in vivo*, it might also prevent MC accumulation at sites affected. CD9 has been described to expose a conformation dependent epitope when clustered with activated β_1-integrins. A mAb directed against this epitope could mimic the PI3K activation (Gutierrez-Lopez et al., 2003). Hence it would be interesting to know if this epitope is also involved in IL-16 induced PI3K signaling by CD9.

CD63

CD63 (TSPAN30, Granulophysin, Melanoma Antigen ME491, Platelet Glycoprotein 40 kDa, sometimes ambiguously lamp-3) was the first tetraspanin that has been cloned in 1988 from human cancer cells (Hotta et al., 1988). Three years later it was already demonstrated to be located in the vicinity of the FcεRI on RBL-2H3 basophils (Kitani et al., 1991). If used in high concentrations, the antibody directed against CD63 moderately inhibited degranulation. In experiments with RBL cells transfected with human CD63, two out of five mAb clones specific for hCD63 stimulated degranulation in the absence of any further stimuli. The addition of a secondary antibody did not further increase degranulation, arguing against a mechanism of mere crosslinking (Smith et al., 1995). The authors of these studies thus speculated that CD63 might be a mediator or promoter of FcεRI signaling, but at that time, no experimental evidence supported this hypothesis. Instead it had been shown intracellularly to co-localize with lamp-1 and lamp-2 on lysosomes (Metzelaar et al., 1991) and on endosomes. Importantly, CD63 is also present on various kinds of secretory granules. It has been detected on basophil granules (Knol et al., 1991), but also on platelet granules (Nishibori et al., 1993), the granzyme/perforin containing granules of cytotoxic T cells (Peters et al., 1991) and the von Willebrand Factor/P-selectin containing Weibel Palade bodies of endothelial cells (Vischer and Wagner, 1993). CD63 is targeted to lysosomes by C-terminal GYEVM motif that is recognized by the adaptor protein AP-3 subunit μ3 (Rous et al., 2002) and has been found to be cycling between endocytic vesicles and secretory granules (Kobayashi et al., 2000).

In the event of granule exocytosis, CD63 containing granule membrane fuses with the plasma membrane. Therefore, CD63 soon was used extensively as activation marker of basophils (Knol et al., 1991) and MC (reviewed in Valent et al., 2001). Close spatial examination of the site of $CD63^+$ vesicle exocytosis showed that it is roughly targeted into the direction of the stimulus, but spares the site of FcεRI crosslinking. This is in contrast to $CD63^-$ recycling endosomes, which are also exocytosed in response to FcεRI activation and are targeted directly to the site of the stimulus (Wu et al., 2007). When additional activation markers were uncovered, we defined a group of activation markers that behaved like CD63, consisting of CD63 and CD107a and indicating anaphylactic exocytosis of large granules. The surface exposure time course of

these "slow" activation markers parallels that of histamine release, in contrast to the "fast" CD203c group (CD203c, CD13, CD64) that rather indicates piecemeal degranulation (Hennersdorf et al., 2005).

However, even under steady state conditions many types of human MC showed constitutive CD63 surface expression. This included skin MC, renal MC, tonsillar MC, lung MC, uterus MC, and gastrointestinal MC as well as cultured MC progenitors from cord blood and basophils (Fureder et al., 1997; Beil et al., 1998; Ghannadan et al., 1998; Krauth et al., 2005; Schernthaner et al., 2005). Furthermore, patients with indolent systemic mastocytosis showed upregulated CD63 on MC (Escribano et al., 1998).

Interactions of various integrins and tetraspanins have been observed in many cell types. Tetraspanins have been shown to be important for integrin mediated intercellular adhesion, regulate integrin mediated cell motility and are involved in integrin signaling (Berditchevski, 2001). CD63 is no exception from the rule that integrins are classical interaction partners of tetraspanins. For example, CD63 has been found associated with LFA-1 (CD11a/CD18) and some mAb against CD63 triggered increased cell adhesiveness probably resulting from Src kinase activation (Skubitz et al., 1996). Recently, in a very similar finding interaction of CD63 and β_1-Integrin triggered by their natural ligand ameloblastin resulted in Src activation as well (Iizuka et al., 2011). In a major advance in the quest for the role of CD63 in MC with a blocking mAb directed against CD63 not only MC adhesion to fibronectin and vitronectin could be inhibited but also FcεRI mediated degranulation of adherent MC. Furthermore, the Ab was sufficient to inhibit FcεRI mediated degranulation in rats. While no effect on tyrosine phosphorylation was observed, the Gab2 PI_3K pathway known to affect adhesion as well as degranulation was shown to be suppressed (Kraft et al., 2005).

Work focusing on intracellular CD63 showed co-localization of CD63 with the SNARE proteins VAMP-7 and syntaxin 3 together with *N*-ethylmaleimide-sensitive factor (NSF; Puri et al., 2003). SNARES are proteins mediating the fusion of granule membrane with the plasma membrane and NSF is an ATPase initiating SNARE disassembly (reviewed by Benhamou and Blank, 2010). SNARE disassembly "primes" MC, enabling further degranulation events. Recently, a mAb was developed that specifically recognizes CD63 of granular origin. Specificity was found to depend on a glucosylation pattern only present on the intracellular isoform. Recognizing CD63 that had reached the cell membrane during a previous degranulation event, this Ab proved that MC can degranulate at least a second time in response to a different IgE-antigen stimulus (Schafer et al., 2010). These findings are complemented by a report that investigated the superior stimulus provided by particulate antigens (pAg). The authors show long lasting interaction of pAg/IgE/FcεRI in $CD63^+$ granule compartments that did not occur when soluble antigen was administered instead (Jin et al., 2011).

New findings show involvement of the autophagy pathway in degranulation and co-localization of CD63 with the autophagy marker LCR-II (Ushio et al., 2011).

The new mAb recognizing granular CD63 will be an important tool in MC research and might also be useful as a diagnostic tool. The capacity of the CD63 blocking antibody to block MC activation *in vivo* might make it a lead for a novel therapy targeting MC granule release. However, as observed with the Hermansky Pudlak syndrome, a rare hereditary disease shown to be the result of CD63 expression deficiency (Nishibori et al., 1993) high dose application of anti-CD63 might negatively affect blood coagulation. The interesting finding that CD63 promotes FcεRI signaling only of adherent MC prompts the question whether integrin binding might effect an activating conformation change in associated CD63 as has been shown for CD9 (Gutierrez-Lopez et al., 2003), thereby amplifying intracellular FcεRI signaling.

CD81

CD81 (TSPAN28, TAPA-1) was identified as the target of an anti-proliferative antibody with strong cDNA homology to CD63 (Oren et al., 1990). It is very broadly expressed on hematolymphoid, neuroectodermal, and mesenchymal cells and has been shown to amplify B-cell- and T-cell-receptor signaling (Levy et al., 1998). Similarly a mAb directed against CD81 was found to down-regulate FcεRI mediated degranulation of RBL cells (Fleming et al., 1997). As was later shown in a similar manner for an CD63 specific mAb (Kraft et al., 2005, see above), this treatment failed to inhibit FcεRI-induced tyrosine phosphorylation. Also, calcium mobilization or leukotriene synthesis was not affected (Fleming et al., 1997). While no signaling pathway mediating the anti-CD81 effect could be elucidated, effectiveness of this Ab in curing passive cutaneous anaphylaxis in rats could be demonstrated. CD81 has been shown to associate with VLA-4 and LFA-1 and to activate their adhesiveness in lymphocytes (Levy et al., 1998). Furthermore it was demonstrated to regulate Rac1 activation in cell migration (Quast et al., 2011). Maybe, reduction of allergen induced airway hyperreactivity observed in CD81 deficient mice (Deng et al., 2000) might in part result of a disrupted FcεRI–CD81 interaction that probably regulates FcεRI signaling in wildtype mice. Compared to CD9 and CD63 the knowledge and understanding of the role of CD81 in MC is quite limited. It would be interesting to investigate whether it promotes adhesion and migration also in MC. The potential application of blocking anti-CD81 as an anti-allergic treatment however, might most prominently inhibit B cell antibody production by inhibiting signaling of the CD19 CD81 B-cell-receptor complex (van Zelm et al., 2010).

CD151

Immunophenotyping with a mAb raised against myeloid leukemia cells identified CD151 (TSPAN24, gp27, PETA-3, Raph blood group) on platelets and megakaryocytes as well as on various types of myeloid, endothelial and epithelial cells (Ashman et al., 1991; Sincock et al., 1997). The examination of CD151 on MC was not as comprehensive as with other tetraspanins, however it has been found on human foreskin, gastrointestinal, uterine, lung, and cord blood derived MC as well as on basophils (Ghannadan et al., 1998; Wimazal et al., 1999; Krauth et al., 2005; Schernthaner et al., 2005). It is well established that CD151 is co-expressed and co-localized with laminin binding integrins, facilitating adhesion, and motility processes, the latter possibly by mediating TEM endocytosis and recycling (Liu et al., 2007). However, there are no functional data regarding the role of CD151

Table 1 | General molecular and prominent functional properties of tetraspanins also expressed in MC.

Name: CD, HGNC; OMIM No.	Gene: accession, location	Protein: accession, size	Function (in human or murine cells)
CD9, TSPAN29; *143030	**Human:** *928, 12p13.3 **Mouse:** *125276 F3	**Human:** NP_001760, 228 aa, 25.4 kDa **Mouse:** NP_031683, 226 aa, 25.3 kDa	Fusion of gametes (Kaji et al., 2000), myoblasts (Tachibana and Hemler, 1999), and virus infected cells (Loffler et al., 1997); represses tumor cell motility (Miyake et al., 1991; Ikeyama et al., 1993); prevents monocyte fusion (Takeda et al., 2003); formation of paranodal nerve junctions (Ishibashi et al., 2004); MHC II multimerization (Unternaehrer et al., 2007). In MC: IL-16 receptor in MC and other cells (Qi et al., 2006); might promote FcεRI (Peng et al., 2011) and FcγR signaling (Huang et al., 1992).
CD63, TSPAN30; *155740	**Human:** *967, 12q12-q13 **Mouse:** *1251210 D3	**Human A:** NP_001771, 238 aa, 25.6 kDa; **Human B:** NP_001035123, 236 aa, 25.4 kDa **Mouse:** NP_031679, 238 aa, 25.8 kDa	Promotes tumor cell motility and migration (Radford et al., 1997); regulates endosomal sorting (van Niel et al., 2011); cofactor for leukocyte recruitment by endothelial P-selectin (Doyle et al., 2011); internalization of H,K-ATPase beta-subunit in gastric parietal cells (Duffield et al., 2003); neutrophil activation and adhesion (Skubitz et al., 1996). Promotes MC adhesion and degranulation (Kraft et al., 2005).
CD81, TSPAN28; *186845	**Human:** *975, 11p15.5 **Mouse:** *125207 F5	**Human:** NP_004347, 236 aa, 25.8 kDa **Mouse:** NP_598416, 236 aa, 25.8 kDa	Promotes CD19 expression/antibody production (van Zelm et al., 2010); T-cell adhesion and co-stimulation (Todd et al., 1996; Sagi et al., 2012); cell migration (Quast et al., 2011); virus entry (Pileri et al., 1998); *Plasmodium* infection (Silvie et al., 2003); prevents monocyte fusion (Takeda et al., 2003); Promotes FcεRI dependent degranulation (Fleming et al., 1997).
CD151, TSPAN24; *602243	**Human:** *977, 11p15.5 **Mouse:** *124767 F5	**Human:** NP_004348, NP_620599[$], 253 aa, 28.3 kDa **Mouse:** NP_001104520, 253 aa, 28.3 kDa	Regulates integrin traffic and promotes cell migration (Liu et al., 2007); enables platelet aggregation by integrin outside-in signaling (Lau et al., 2004); strengthens adhesion to laminin-1 (Lammerding et al., 2003); inhibits T-cell proliferation (Wright et al., 2004); strengthens podocyte binding to glomerular basement membrane (Sachs et al., 2012).

Accession numbers of the respective NCBI databases (OMIM, Gene, or Protein reference sequence) are given. [$]Results of a transcript that lacks an alternate segment in the 5′ UTR.

in MC yet. The data from other cell types, however, suggest it to be an integral TEM component. Thus it is unlikely, that CD151 present on MC is devoid of functions regarding intercellular binding, migration, and recycling of TEM membrane patches.

CONCLUDING REMARKS

Tetraspanins are closely linked to MC biology. While CD63 is commonly used as an activation marker for human cells and co-localization of tetraspanins with FcεRI has been known for a long time, it has been much more difficult to gain functional insights. While knowledge about the modes of MC tetraspanin action is increasing recently, there are very few results generated with primary cells and little attention has been paid to the fact that the tetraspanins expressed in MC might be redundant in function. Anyway, it is becoming increasingly clear that tetraspanins form functional clusters with Fcε receptors and integrins on MC surfaces and connect them with downstream signaling components like Src or Gab2.

The development of novel mAb that specifically recognize CD63 of intracellular origin (Schafer et al., 2010) or that are able to attenuate MC degranulation by targeting CD63 or CD81 (Fleming et al., 1997; Kraft et al., 2005) might improve diagnostics and provide the basis to develop powerful tools for the treatment of acute allergic reactions. Furthermore, tetraspanins might play a key role in emerging topics of MC research: (1) MC are regarded as important sources of TNF under pro-inflammatory conditions (Biedermann et al., 2000; Kneilling et al., 2009) and tetraspanins might contribute to metallo-protease cleavage of pro-TNF as has been described for other cells (Arduise et al., 2008). (2) Tetraspanins might be involved in establishing mast cell adhesion and cell–cell contacts to a much larger extent than anticipated today (Kraft et al., 2005). (3) MC are increasingly perceived as antigen presenting cells (Kambayashi et al., 2009; Stelekati et al., 2009), thus it is of interest if the effects of tetraspanins on antigen presentation reported from dendritic cells and B cells might also be observed in MC. (4) Recent research demonstrated MC as a source of exosomes (Skokos et al., 2002) of which tetraspanins

are major membrane components. As has been described for exosomes of many other cells types, MC exosomes stained positive for CD63 (Valadi et al., 2007). Thus, adding to tetraspanin research on MC plasma membrane or MC granules, MC exosomes open an additional new field for the examination of MC tetraspanins.

All together, accumulating evidence suggests that tetraspanins are hitherto underrated contributors in the regulation of effector functions also in MC. Although we are still missing some important data on tetraspanin functions in MC, already our present understanding highlights that tetraspanins may be relevant druggable targets in future therapeutic strategies to treat allergic and possibly also other MC-dependent diseases.

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Mast cell chemotaxis – chemoattractants and signaling pathways

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Migration of mast cells is essential for their recruitment within target tissues where they play an important role in innate and adaptive immune responses. These processes rely on the ability of mast cells to recognize appropriate chemotactic stimuli and react to them by a chemotactic response. Another level of intercellular communication is attained by production of chemoattractants by activated mast cells, which results in accumulation of mast cells and other hematopoietic cells at the sites of inflammation. Mast cells express numerous surface receptors for various ligands with properties of potent chemoattractants. They include the stem cell factor (SCF) recognized by c-Kit, antigen, which binds to immunoglobulin E (IgE) anchored to the high affinity IgE receptor (FcεRI), highly cytokinergic (HC) IgE recognized by FcεRI, lipid mediator sphingosine-1-phosphate (S1P), which binds to G protein-coupled receptors (GPCRs). Other large groups of chemoattractants are eicosanoids [prostaglandin E_2 and D_2, leukotriene (LT) B_4, LTD_4, and LTC_4, and others] and chemokines (CC, CXC, C, and CX3C), which also bind to various GPCRs. Further noteworthy chemoattractants are isoforms of transforming growth factor (TGF) β1–3, which are sensitively recognized by TGF-β serine/threonine type I and II β receptors, adenosine, C1q, C3a, and C5a components of the complement, 5-hydroxytryptamine, neuroendocrine peptide catestatin, tumor necrosis factor-α, and others. Here we discuss the major types of chemoattractants recognized by mast cells, their target receptors, as well as signaling pathways they utilize. We also briefly deal with methods used for studies of mast cell chemotaxis and with ways of how these studies profited from the results obtained in other cellular systems.

Keywords: mast cell, IgE receptor, plasma membrane, chemoattractant, chemotaxis, cell migration, signal transduction

INTRODUCTION

Mast cells are derived from progenitors that are released from bone marrow into circulation, and subsequently migrate into peripheral tissues. Within this time period they undergo differentiation and maturation. These processes are controlled by chemotactic agents and growth factors present in circulation and at sites of mast cell residency. In peripheral tissues the progenitors differentiate into one of two possible classes of mature mast cells, connective tissue, or mucosal mast cells. They differ in many properties, including the mediators they produce and secrete. Connective tissue mast cells are found in the skin, around blood vessels, and in peritoneal cavity, whereas mucosal mast cells localize in the linings of the lung and intestine. Differentiation of mast cells into these two classes is directed by various growth factors and chemoattractants produced by various cell types present in the surrounding tissue environment. Accumulation of mast cells within tissues is observed in many pathophysiological conditions. Mature mast cells infiltrate the sites of inflammation associated with chronic atopic disease or during helminthic or bacterial infection. Directed migration of mature mast cells or their progenitors might be one of the key mechanisms responsible for local accumulation of these cells. This recruitment depends on the presence of chemical compounds named chemoattractants, which are produced locally at sites of inflammation by various cell types. Importantly, mast cells themselves can produce and secrete various chemoattractants including adenosine, sphingosine-1-phosphate (S1P), and leukotriene (LT) B_4, and attract in this way other mast cells and/or their progenitors by the autocrine/paracrine fashion. Various chemoattractants and the corresponding receptors are involved in mast cell migration and they can in part share the downstream signaling pathways. Others use pathways that are unique for each particular chemoattractant/receptor. This divergence and convergence of signaling pathways is important for proper cooperation among receptors triggered with various chemoattractants, and contributes to specific behavior of mast cells. There are two main types of migration, chemokinesis and chemotaxis. Whereas chemokinesis is a random, non-vectorial moiety, chemotaxis is directional migration toward higher concentrations of a chemoattractant. This review deals with major types of mast cell chemoattractants and their production, as well as with signaling pathways they utilize. It starts with a brief description of methods used for studies of mast cell chemotaxis.

METHODS USED FOR STUDIES OF MAST CELL CHEMOTAXIS

Numerous methods have been developed for studies on mast cell migration, chemotaxis, and activity of various cytokines. Although there are large numbers of mast cells in an adult organism, they cannot be easily isolated because they are scattered throughout

various parts of the body. In most of the studies, mast cells of mouse, rat, and human origin have been used. In mice, bone marrow derived mast cells (BMMCs) are often studied. These cells can be obtained by culturing bone marrow progenitors in the presence of interleukin (IL)-3 and SCF (Razin et al., 1981; Tsai et al., 1991). In rat, mast cells are often isolated in mature stage from peritoneal cavity (Cooper and Stanworth, 1976; Poole and Zetter, 1983; Gruber et al., 1994; Brzezińska-Błaszczyk et al., 2007). Human mast cells are usually obtained by culturing mast cell progenitors from various tissues in the presence of SCF, IL-6, and IL-10. Progenitors from skin give rise to skin mast cells (SMCs; de Paulis et al., 2001); those from intestine produce intestine mast cells (IMCs; Fox et al., 1985; Feuser et al., 2012); progenitors from lungs, cord blood, or peripheral blood differentiate, respectively into lung mast cells (LMCs; Romagnani et al., 1999; Brightling et al., 2005b), cord blood mast cells (CBMCs; Nilsson et al., 1994; Ochi et al., 1999; Oskeritzian et al., 2008), or peripheral blood mast cells (PBMCs; Saito et al., 2006; Aung et al., 2011). Human BMMCs have been also used in some studies (Brightling et al., 2005b). Chemotaxis was also studied using mast cell lines, such as rat basophilic leukemia (RBL) cells, clone 2H3 (Jolly et al., 2004, 2005; Toda et al., 2004), human mast cell line (HMC-1; Nilsson et al., 2000; Brightling et al., 2005b; Lundeen et al., 2006; and others), or human lymphoma cell line LAD-2 (Oskeritzian et al., 2008).

Experiments with various mast cell types have shown that the expression pattern of surface receptors for chemoattractants depends on origin of the cells and their maturation (Ochi et al., 1999; Weller et al., 2005; Collington et al., 2010a). Results on the effect of chemoattractants also depend on the method used. The methods can be divided on methods *in vivo* and *in vitro*.

METHODS *IN VIVO*

Methods *in vivo* usually evaluate the accumulation of mast cells at sites of chemoattractant injection. Chemoattractants are usually injected intradermally (i.d.) and mice are sacrificed at various time intervals after injection. Skin at the site of injection is then removed, fixed, stained with toluidine blue, and examined by microscopy to determine the number of mast cells (Matsui and Nishikawa, 2005; Kitaura et al., 2005b).

Alternatively, mast cell progenitors or mature mast cells are isolated and cultured up to definite developmental stages. The cells are then labeled with various trackers (fluorescent or radioactive) followed by intravenous (i.v.) tail vein injection some time before i.d. injection of chemoattractant into dorsal skin. The mice are then sacrificed and skin biopsies are evaluated depending on the tracker used (Weller et al., 2005, 2007; Boehme et al., 2009; Collington et al., 2010a). In such experiments, mast cells obtained from mice deficient in specific genes could be injected into mice deficient in mast cells to determine the role of selected molecules in chemotaxis. Exposure of the cells to antibodies specific for selected surface receptors can be used for determination of the possible role of the receptors in mast cell chemotaxis (Brightling et al., 2005b; Kitaura et al., 2005b; Kuehn et al., 2010).

METHODS *IN VITRO*

Most of the assays on chemotaxis *in vitro* utilized various modifications of Boyden's chamber where cells migrate toward chemoattractants through pores (usually 5 or 8 μm) of polycarbonate membrane. Common is the use of Transwell permeable supports placed into 24-well polystyrene plates. Mast cells are introduced into the upper chamber, which is placed into a well containing chemotaxis buffer supplemented with the chemoattractant at selected concentration. The plates are kept for several hours (usually 2–8) at 37°C in CO_2 incubator. The cells migrate toward chemoattractant through the pores of the membrane and accumulate at the bottom of the well. The number of cells is counted with a hemocytometer or flow cytometer. Alternatively, the cells are labeled with fluorescent dye and quantified by determining the florescence (Weller et al., 2005; Kuehn et al., 2010; Tůmová et al., 2010).

In some experiments the membranes were modified by coating with different substances, such as laminin or fibronectin, or epithelial cells (Nilsson et al., 1994; Oliveira and Lukacs, 2001; Kitaura et al., 2005b) and migration was evaluated by counting the number of migrated cells. Boyden chamber-based assays can also be used for identification of molecules or signaling pathways involved in chemotaxis. One major advantage of this system is its simplicity and ability to test many compounds at different concentrations simultaneously, to examine the role of pharmacological inhibitors on chemotaxis, and also to analyze chemokinesis, which reflects migration independent on the presence of chemoattractants. However, this method has its limitation. One of them is that only sharp chemotactic gradient can be generated and it is impossible to assess the speed of migration of individual cells or directions of movement of the cells.

Methods analyzing individual migrating cells in real time are often based on microscopic technique and on recording the cell movement in selected time intervals. These real time methods could be combined with different modifications of under-agarose or "tunnel" assays, which are used for studying multiple chemotactic gradients, shape and speed of migrating cells, or assessing the roles of selected adhesion molecules (Poole and Zetter, 1983; Heit et al., 2002). In the under-agarose method, tissue culture dishes are precoated with a blocking agent such as BSA, calf serum, or collagen, and then overlaid by agarose. After agarose solidifies, three holes of 3 mm in diameter and 3 mm apart are punched in the gel. Mast cells are dispensed to the middle hole and chemoattractants to the others. The migration is observed under microscope in real time or the migrated cells can be stained at the end of the assay and quantified. This method can also be modified by using specific inhibitors, activators, or antibodies (Heit et al., 2002). The obtained images can by analyzed by cell tracking plugins: http://rsb.info.nih.gov/ij/; http://www.imagescience.org/meijering/software/mtrackj; or http://www.ibidi.com/applications/ap_chemo.html

Horizontal chemotactic assays in KK chambers (Kanegasaki et al., 2003) were used for studying the migration of BMMCs toward antigen. KK chambers consist of etched silicon substrate and a flat glass plate that forms compartment with a 5-μm-deep microchannel. A charge-coupled device camera is used to record the migrating cells (Kanegasaki et al., 2003; Sawada et al., 2005). The main advantage of recording the cells in real time is that investigators can observe not only individual migrating cells but also their dynamic behavior during the process. This method

in combination with cells carrying fluorescently tagged proteins could also be useful for studying the involvement of these proteins in chemotaxis.

MAST CELL CHEMOATTRACTANTS

Numerous chemoattractants have been described capable of inducing chemotaxis in mast cells. Some of them, and corresponding receptors, are summarized in **Table 1** and described below.

STEM CELL FACTOR

Stem cell factor, also known as steel factor or c-Kit-ligand, is a hematopoietic growth factor that promotes survival, proliferation, and differentiation of hematopoietic cells (for review see Roskoski, 2005; Okayama and Kawakami, 2006; Jensen et al., 2008). It is produced in both soluble and membrane-bound form by alternative splicing of the same RNA transcript, and is a major chemotactic factor for mast cells and their progenitors (Chabot et al., 1988; Meininger et al., 1992; Nilsson et al., 1994, 1998). SCF is produced by a wide variety of cells including fibroblasts and endothelial cells. Its receptor, c-Kit, is a type III tyrosine kinase broadly expressed on mature mast cells and eosinophils. SCF promotes recruitment of mast cell progenitors into tissues, as well as their local maturation and activation. It also promotes eosinophil survival, maturation, and functional activation (Chabot et al., 1988; Okayama and Kawakami, 2006). Binding of SCF to the cell induces dimerization of the receptors, followed by their transphosphorylation at tyrosine residues (Tyr568 and Tyr570) and consequently formation of docking sites for the Src-homology (SH) 2-containing signal transduction molecules. It has been demonstrated that Src-family protein tyrosine kinases Lyn and Fyn are phosphorylated and activated after c-Kit triggering (Linnekin et al., 1997; Timokhina et al., 1998) and that the event leads to further propagation of the signal (**Figure 1**).

There are several signaling pathways resulting in degranulation, survival, and migration of mast cells. An important pathway depends on PI3K and subsequent phosphorylation of Akt, and is therefore related to c-Kit-dependent mast cell survival. Fyn-dependent axis leads to the phosphorylation of Gab2, mediates through small GTPase Rac the cytoskeleton reorganization and influences mast cell migration (Linnekin et al., 1997; Timokhina et al., 1998; Samayawardhena et al., 2006; Samayawardhena et al., 2007). Studies with different murine c-Kit mutants showed the importance of Y719 and Y567 for c-Kit-mediated chemotaxis. Phosphorylated Y719 recruited PI3K and mediated thus an enhanced Ca^{2+} signal, which was found to be critical for chemotaxis. In contrast, phosphorylated Y567 recruited Lyn or Fyn resulting in activation of p38 pathway, also important for chemotaxis (Ueda et al., 2002; Samayawardhena et al., 2006). Decreased migration toward SCF was observed in cells with a defect in expression of protein tyrosine phosphatase (PTPα; Samayawardhena and Pallen, 2008). When compared to wild-type cells, the PTPα-deficient cells exhibited reduced Fyn kinase activity causing defects in phosphorylation of tyrosines 567/569 and 719 of c-Kit. PI3K and Akt activation was unaffected in PTPα$^{-/-}$ BMMCs. Thus PTPα is required for SCF-induced migration which employs the Fyn/Gab2/Shp2/Vav/PAK/Rac/JNK signaling axis (Samayawardhena and Pallen, 2008). Chemotaxis is also positively regulated by Fes kinase because migration of Fes$^{-/-}$ mast cells toward SCF was decreased (Smith et al., 2010).

Studies with macrophages showed regulation of PI3K-dependent migration by negative feedback of phosphatase and tensin homolog (PTEN; Papakonstanti et al., 2007). Knock-down of PTEN in mast cells led to increased basal level of PIP3 and constitutive activation of Akt, p38, and JNK, resulting in enhanced survival and increased production of several cytokines, including IL-3, IL-6, and tumor necrosis factor (TNF)-α in antigen-activated cells (Furumoto et al., 2006). It was also shown that PTEN deficiency enhanced the number of mast cells in different tissues. This suggested that PTEN could play a regulatory role in mast cell chemotaxis (Furumoto et al., 2006). Experiments with neutrophils showed that SHIP1, other phosphatase regulating amount of PIP3, was even more important for migration than PTEN (Nishio et al., 2007; Subramanian et al., 2007). Interestingly, SHIP1 knock-out mice exhibited mast cell hyperplasia in several tissues (Haddon et al., 2009), which could be also consequence of increased chemotaxis. Elucidation of the role of these two phosphatases in mast cell chemotaxis requires further studies.

Mammalian target of rapamycin complexes (mTORCs) were found to play important roles in chemotaxis of several cell models such as neutrophils (Charest et al., 2010; Liu et al., 2010) and *Dictyostelium* (Sasaki and Firtel, 2006; Takeda et al., 2007; Liu and Parent, 2011). mTORC1 is activated in PI3K-dependent manner and its inhibition by rapamycin depressed the SCF-mediated migration (Kim et al., 2008a,b). mTORC2 appears to play an important role in PGE_2-mediated chemotaxis (Kuehn et al., 2011b; see below) but its role in SCF- or antigen-mediated chemotaxis is to be defined.

In this connection, it should be mentioned that patients with c-Kit mutation D816V exhibit constitutive activation of c-Kit and accumulation of mast cells derived from CD34$^+$CD117$^+$ mast cell precursors. Experiments with such precursors obtained from patients with mastocytosis showed that only less than 10% prechemotactic sample had D816V mutation, whereas as many as 40–80% of migrated cells showed the mutation (Taylor et al., 2001). The results indicate that D816V mutation in c-Kit enhances the SCF-dependent chemotaxis and could promote in this way the mastocytosis.

ANTIGEN

FcεRI is a tetrameric receptor consisting of an immunoglobulin E (IgE)-binding α chain, β chain, and two γ chains. Binding of IgE to α chain and subsequent crosslinking of the receptor by the multivalent antigen leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the FcεRI β and γ chains by Lyn kinase (**Figure 2**; Eiseman and Bolen, 1992; Yamashita et al., 1994). Phosphorylated ITAMs are able to bind a broad variety of positive as well as negative regulators of immunoreceptor signaling. The most important propagator of the positive signal is a protein tyrosine kinase Syk, which after binding to phosphorylated tyrosines of ITAM assumes an active conformation facilitating its phosphorylation by Lyn and further increase in enzymatic activity. Consequently, Syk phosphorylates a number of its downstream targets critical for further

Table 1 | Summary table of different mast cell chemoattractants and their receptors.

Chemoattractant*	Receptor	Mast cell model (organism used)	Reference
SCF	c-KIT (CD117)	BMMCs (mouse), PMCs (mouse), CBMCs (human), HMC-1 (cell line, human)	Meininger et al. (1992), Nilsson et al. (1994), Samayawardhena et al. (2006)
Antigen, HC IgE	FCεRI	MC-9 mast cell clone (mouse), BMMCs (mouse)	Ishizuka et al. (2001), Kitaura et al. (2005b), Tùmová et al. (2010)
S1P	S1PR1, S1PR2	BMMCs (mouse), LAD-2 (cell line, human), CBMCs (human), RBL-2H3 (cell line, rat)	Olivera et al. (2006), Oskeritzian et al. (2008), Jolly et al. (2004, 2005)
PGE_2	EP3	BMMCs (mouse)	Weller et al. (2007), Kuehn et al. (2011b)
PGD_2	DP1, DP2 (CRTh2)	BMMCs (mouse)	Lewis et al. (1982), Boehme et al. (2009)
LTB_4	BLT1, BLT2	BMMCs (mouse), HMC-1 (cell line, human), CBMCs (human)	Lundeen et al. (2006), Weller et al. (2005)
LTD_4, LTC_4	CysLT1R, CysLT2R	$CD34^+$ hematopoietic progenitors (human)	Bautz et al. (2001)
CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL14 (HCC-1), CCL15 (MIP-1δ), CCL16 (HCC-4), CCL23 (MPIF-1)	CCR1	LMCs (human), BMMCs (human), CBMCs (human), RBL-2H3 (cell line, rat)	Brightling et al. (2005b), Juremalm et al. (2002), Scott and Bradding (2005), Toda et al. (2004)
CCL 5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL11 (eotaxin), CCL13 (MCP-4), CCL15 (MIP-1δ), CCL24 (eotaxin-2), CCL26 (eotaxin-3), CCL28 (MEC)	CCR3	LMCs (human), BMMCs (human), CBMCs (progenitors, human), SMC (human)	Brightling et al. (2005b), Ochi et al. (1999), Romagnani et al. (1999), Romagnani (2002), de Paulis et al. (2001), Scott and Bradding (2005)
CCL5 (RANTES)	CCR4	LMCs (human), CBMCs (human)	Brightling et al. (2005b), Juremalm et al. (2002)
CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (eotaxin)	CCR5	CBMCs (progenitors, human)	Ochi et al. (1999), Scott and Bradding (2005)
CCL16 (HCC-4), CCL20	CCR6	IMCs (human)	Feuser et al. (2012)
CCL19 (MIP-3β), CCL21 (6Ckine)	CCR7	LMCs (human), BMMCs (human) IMCs (human)	Brightling et al. (2005b), Feuser et al. (2012)
CCL1	CCR8	IMCs (human)	Feuser et al. (2012)
CXCL6 (GCP-2), CXCL8 (IL-8)	CXCR1	LMCs (human), BMMCs (human), CBMCs (human) IMCs (human)	Brightling et al. (2005b), Inamura et al. (2002), Feuser et al. (2012)
CXCL8 (IL-8)	CXCR2	CBMCs (human) IMCs (human)	Inamura et al. (2002), Ochi et al. (1999), Feuser et al. (2012)
CXCL9 (Mig), CXCL10 (IP-10), CXCL11 (I-TAC)	CXCR3	CBMCs (progenitors and mature, human), synovial MCs (human), LMCs (human), HMC-1 (cell line, human), IMCs (human)	Ochi et al. (1999), Ruschpler et al. (2003), Brightling et al. (2005a), Feuser et al. (2012)
CXCL12 (SDF-1α)	CXCR4	LMCs (human), BMMCs (human), HMC-1 (cell line, human), CBMCs (progenitors, human), IMCs (human), BMMC (mouse	Brightling et al. (2005b), Ochi et al. (1999), Feuser et al. (2012), Byrne et al. (2008)
CXCL16 (GCP-2)	CXCR6	LMCs (human), BMMCs (human), IMCs (human)	Scott and Bradding (2005), Brightling et al. (2005b), Feuser et al. (2012)

(Continued)

Table 1 | Continued

Chemoattractant*	Receptor	Mast cell model (organism used)	Reference
CX3CL1 (fractalkine)	CX3CR1	BMMCs (mouse), IMCs (human)	Papadopoulos et al. (2000), Feuser et al. (2012)
TGF-β	TGF-β I and II receptors	CMC3 (cell line, mouse), PMCs (rat), HMC-1 (cell line, human), CBMCs (human), MCs (mouse)	Gruber et al. (1994), Olsson et al. (2000), Matsui and Nishikawa (2005)
Adenosine	Adenosine receptors	BMMCs (mouse)	Kuehn et al. (2010)
C1q	cC1q-R, gC1q-R	C57 (cell line, mouse), HMC-1 (cell line, human)	Ghebrehiwet et al. (1995)
C3a	C3AR1	HMC-1 (cell line, human), CBMCs (human), SMCs (human)	Hartmann et al. (1997), Nilsson et al. (1996)
C5a	C5AR	HMC-1 (cell line, human), CBMCs (human), SMCs (human)	Hartmann et al. (1997), Nilsson et al. (1996)
PAF	PAF-receptor	HMC-1 (cell line, human), BMMCs (mouse), CBMCs (human)	Nilsson et al. (2000)
5-hydroxytryptamine	5-HT receptors	BMMCs (mouse), PBMCs (human)	Kushnir-Sukhov et al. (2006)
Catestatin	**	LAD-2 (cell line, human), PBMCs (human)	Aung et al. (2011)
TNF-α	TNF-α receptor	PMC (rats)	Brzezińska-Błaszczyk et al. (2007), Misiak-Tloczek and Brzezińska-Blaszczyk (2009)
Glycyl-histidyl-lysine, *N*-formyl-methionyl-leucyl-phenylalanine (tumor derived peptides)	**	PMC (rats)	Poole and Zetter (1983)
Adrenomedullin	**	HMC-1	Zudaire et al. (2006)

**For chemokines both new and old (in parenthesis) names are used in accord to chemokine nomenclature (Zlotnik and Yoshie, 2000).*

***Specific receptors for the chemoattractants in mast cells have not yet been identified.*

PMCs, peritoneal mast cells; RANTES, regulated upon activation, normal T-cell expressed and secreted.

propagation of the signal. These targets include transmembrane adaptor proteins, linker of activated T cells (LAT), and non-T cell activation linker (NTAL), as well as cytosolic adaptor proteins, such as growth factor receptor-bound protein 2 (Grb2), Grb2-associated binder (Gab2), Grb2-related adaptor downstream of Shc (Gads), SH2 domain containing leukocyte protein of 76 kDa (SLP-76) and others that often function in cooperation with various signaling proteins, forming multicomponent signaling units, signalosomes. Phosphorylated LAT associates directly or indirectly with numerous signaling molecules such as Grb2, phospholipase C γ1 (PLCγ1), guanine nucleotide exchange factor Vav, Cbl, SLP-76, and Gads (Gilliland et al., 1992; Buday et al., 1994; Sieh et al., 1994; Trub et al., 1997; Finco et al., 1998; Zhang et al., 1998; Liu et al., 1999; Ishiai et al., 2000). Phosphorylated NTAL interacts with a similar set of proteins except for PLCγ1; this is an important difference explaining in part different roles of these two adaptors in mast cell signaling (Brdička et al., 2002; Janssen et al., 2003; Volná et al., 2004; Zhu et al., 2004; Draber et al., 2012). Further propagation of the signal activates PI3K, PLCγ, protein kinase C (PKC), and mitogen-activated protein kinase (MAPK).

Whereas the signaling pathways leading to degranulation and production of cytokines are relatively well understood, those involved in mast cell migration and chemotaxis are still vague. The first report on molecules involved in antigen-induced mast cell migration showed that p38 MAPK, MAPK-activated protein kinase 2 (MAPKAPK2) and Rho-associated kinase (ROCK) are involved (Ishizuka et al., 2001). Studies with BMMCs isolated from NTAL$^{-/-}$ mice showed an important role of NTAL in antigen-induced migration. Absence of NTAL resulted in enhanced migration toward antigen, when compared to cells from wild-type mice; this negative regulatory role of NTAL is probably mediated by small GTPase RhoA and its kinase ROCK (Tůmová et al., 2010). RhoA regulates cortical filamentous (F)-actin disassembly, which is dependent on enhanced levels of free cytoplasmic calcium (Sullivan et al., 1999). Immunoreceptor activation leads to Ca^{2+} release from intracellular stores, which is followed by entry of external Ca^{2+} into the cells through store operated Ca^{2+} (SOC) channels.

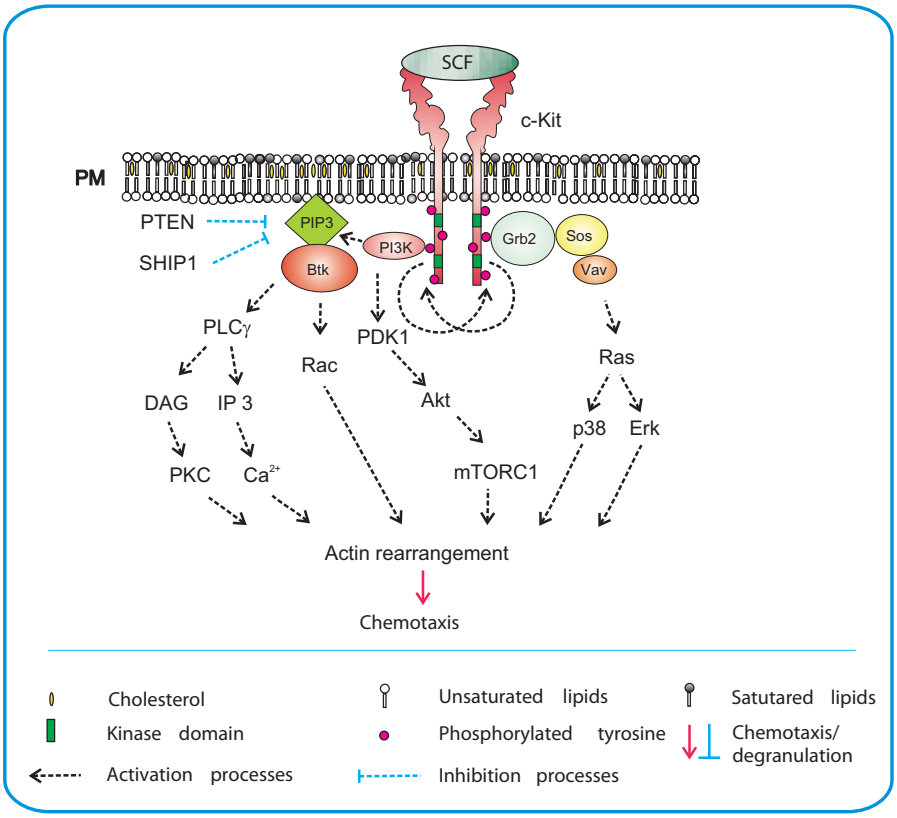

FIGURE 1 | SCF-mediated chemotaxis. Crosslinking of the plasma membrane (PM) anchored c-Kit by SCF results in dimerization of the receptor and its auto-transphosphorylation. This is followed by recruitment of SH2-domain containing proteins such as PI3K and Grb2 to c-Kit. Activated PI3K generates PIP3, a binding site for PH-domain containing protein Btk, and facilitates further propagation of the signal through activation of PLCγ. An increased activity of PLCγ leads to production of DAG and IP3 and release of Ca^{2+}. This is followed by actin rearrangements and chemotactic response. Production of PIP3 also leads to recruitment of phosphoinositide-dependent kinase (PDK)-1 and Akt to the plasma membrane and subsequent phosphorylation of Akt by PDK1. Akt directly phosphorylates the negative regulator of mTORC1, inactivating thereby its inhibitory action. PIP3 levels are negatively regulated by phosphatases PTEN and SHIP1. Grb2 orchestrates activation of Ras by recruiting Ras and Rho family GEFs, Sos, and Vav. Through Ras, both p38 and Erk are activated and chemotaxis is also promoted in this way. Signaling molecules in bodies are those located at their sites of action and/or bound to the indicated target molecules; other signaling molecules are presented as plain text.

SOC influx depends on the presence of aggregated stromal interaction molecule 1 (STIM1; Dráber and Dráberová, 2005; Liou et al., 2005; Roos et al., 2005). The importance of SOC influx in immunoreceptor-directed chemotaxis was recently documented in studies with BMMCs deficient in STIM1. The defect resulted in inhibition of antigen-induced Ca^{2+} response, decreased formation of microtubule protrusions and impaired chemotactic response to antigen (Hájková et al., 2011). Experiments with pharmacological inhibitors showed that both Src and Syk kinases are important for antigen-mediated chemotaxis, whereas Tec kinases are not. These results were confirmed in studies using BMMCs derived from mice deficient in Syk, Btk, Lyn, Fyn, and Hck. Furthermore, the results demonstrated that Lyn is more important than Fyn, and that Hck has no fundamental role in this process. Studies with other inhibitors showed that PI3K, MAP kinases, p38, JNK, and PKC are all essential for antigen-mediated chemotaxis, unlike PKA or Rho-kinase (Kitaura et al., 2005b). The results with Rho-kinase and JNK are, however, in conflict with those presented by Ishizuka et al. (2001).

PI3K appears in several isoforms, and PI3Kδ is the major regulator of chemotactic responses to antigen (Kuehn et al., 2010). In the cited study, the authors also showed the importance of Btk for antigen-mediated migration, which seems to disagree with other published data. Kitaura et al. (2005b) reported that antigen-mediated chemotaxis operates in an autocrine–paracrine manner by releasing other chemoattractants such as monocyte chemoattractant protein (MCP)-1, LTB_4, and adenosine, promoting chemotaxis toward antigen, and that Tec kinases are not involved in this process. These discrepancies could be attributable to different methods used for evaluation of chemotaxis: fibronectin-coated polycarbonate membranes (Kitaura et al., 2005b), against uncoated membranes (Kuehn et al., 2010). It is known that fibronectin binds integrin and that the mechanisms of integrin-mediated migration differ from those of integrin-independent movement (Narumiya and Watanabe, 2009; Renkawitz et al., 2009).

It has been reported that pretreatment of mast cells with SCF leads to inhibition of chemotaxis toward antigen (Sawada et al.,

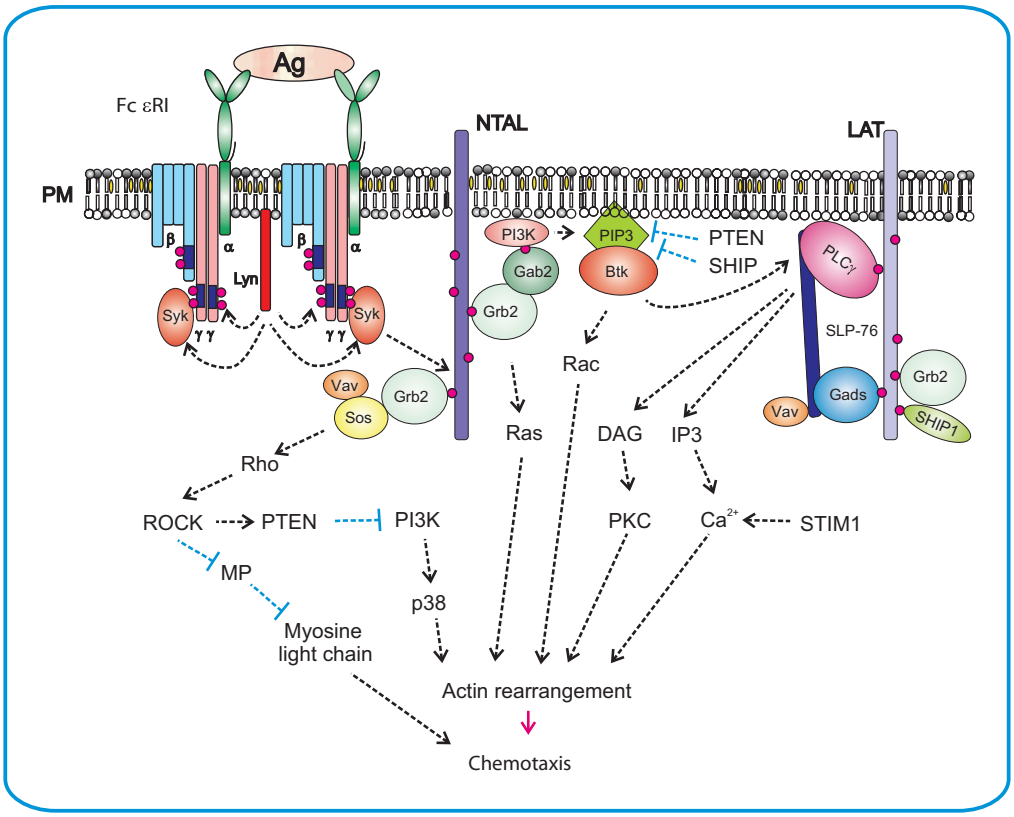

FIGURE 2 | Antigen-mediated chemotaxis. Aggregation of the FcεRI by multivalent antigen leads to rapid Lyn kinase-mediated phosphorylation of tyrosine residues in ITAM motifs of FcεRI β and γ subunits. This is followed by anchoring of Syk to FcεRI γ through interaction of Syk SH2 domains with phosphorylated ITAMs. Syk then phosphorylates transmembrane adaptor proteins NTAL and LAT and creates binding sites for various SH2-containing proteins like Grb2. In this way it brings PI3K and Gab2 to the plasma membrane. Activated PI3K generates PIP3, a binding site for PH-domain containing protein Btk. This leads to further propagation of the signal through activation of PLCγ, resulting in Ca^{2+} release and actin rearrangement. Levels of free intracellular Ca^{2+} are positively regulated by aggregated STIM1. 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/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.

2005). The authors suggested that *in vivo* locally produced SCF may have inhibitory effects on chemotaxis in mast cells with activated FcεRI. This could foster the accumulation of mast cells at sites of inflammatory reactions. In contrast, chemotactic response of cells exposed simultaneously to antigen and SCF was higher than the response toward antigen alone, but lower than that induced by SCF alone (Tùmová et al., 2010). However, different results were obtained in other experiments in which antigen and SCF added together caused a considerably higher chemotactic response than any of them alone (Kuehn et al., 2010). These discrepancies probably reflect differences in cells used, employed assays of migration, and/or other variables.

Antigen-mediated engagement of FcεRI also has a distinct capacity to potentiate the action of PGE_2, adenosine, and probably other chemoattractants (Zhong et al., 2003; Kuehn and Gilfillan, 2007; Kuehn et al., 2008, 2010). The observed crosstalk between signaling pathways that are induced by different activators might play an important role in mast cell physiology.

HIGHLY CYTOKINERGIC IgE

Mast cell activation and migration can also be induced by HC IgE in the absence of antigen (Kitaura et al., 2005a,b). The pathways and molecules involved in this process appear to be similar to those implicated in processes where IgE FcεRI complexes are aggregated by multivalent antigen.

SPHINGOSINE-1-PHOSPHATE

The lipid mediator S1P takes part in diverse biological processes, from vascular, and neural development to regulation of lymphocyte trafficking (Olivera, 2008; Rivera et al., 2008). S1P is produced by intracellular phosphorylation of sphingosine by two phylogenetically conserved sphingosine kinases SphK1 and SphK2 (Olivera and Spiegel, 2001). However, S1P can also be produced extracellularly by the secreted form of SphK1 (Ancellin et al., 2002). Intracellular levels of S1P are tightly regulated by a balance between its synthesis and degradation, occurring either reversibly by two specific S1P phosphatases or irreversibly by S1P lyase. It has

been suggested that S1P lyase and S1P phosphatases can also influence the concentrations of S1P in the interstitium of lymph nodes and spleen, and probably other tissues as well, allowing the cells in those microenvironments to respond to local changes in S1P concentration (Schwab et al., 2005; Peest et al., 2008). Although SphK1 and SphK2 are highly homologous, they are not fully redundant functionally. SphK1 plays a positive role in cell growth and survival (Olivera et al., 1999), whereas overexpression of SphK2 induces cell death (Liu et al., 2003). On the other hand, studies employing genetically manipulated mice showed that knock-outs of either SphK1 or SphK2 separately were viable, had only slightly decreased S1P levels and exhibited no obvious phenotypes, whereas double SphK1/SphK2 knock-outs resulted in a complete loss of S1P and were embryonically lethal. It implies that these kinases have some redundant and overlapping functions (Mizugishi et al., 2005).

Activation of FcεRI in mast cells correlated with increased activity of both SphKs, enhanced the formation of intracellular S1P, and promoted the export of S1P into extracellular space by specific ATP-binding cassette (ABC)-type transporters. After secretion, S1P can act in an autocrine/paracrine-dependent manner. Although both SphK1 and SphK2 have been implicated in regulation of S1P production in mast cells, the details are still obscure. Loss of SphK2 in murine mast cells reduced S1P production and caused a substantial inhibition of FcεRI-mediated degranulation as well as diminished production of IL-6, IL-13, and TNF-α, primarily due to reductions in intracellular calcium levels and PKC activation. In contrast, SphK1-deficiency lowered the level of circulatory S1P *in vivo* and altered mast cell functions (Olivera et al., 2007). SphK1 unlike SphK2 is critical in human mast cells for antigen-induced degranulation, chemoattractants secretion, and migration, while both isozymes are important for cytokine secretion (Oskeritzian et al., 2008). It should be noted that down-regulation of SphK1 also mitigated the rapid and transient increase in intracellular calcium induced by FcεRI crosslinking (Melendez and Khaw, 2002). SphKs phosphorylation, probably by Erk, as well as their recruitment to the proximity of cell membrane close to their substrates, are necessary steps for their complete activation (**Figure 3**). Two Src-family kinases, Lyn and Fyn, play crucial roles in early stages of FcεRI activation and are irreplaceable in activations of SphKs. Lyn was found important for the early onset

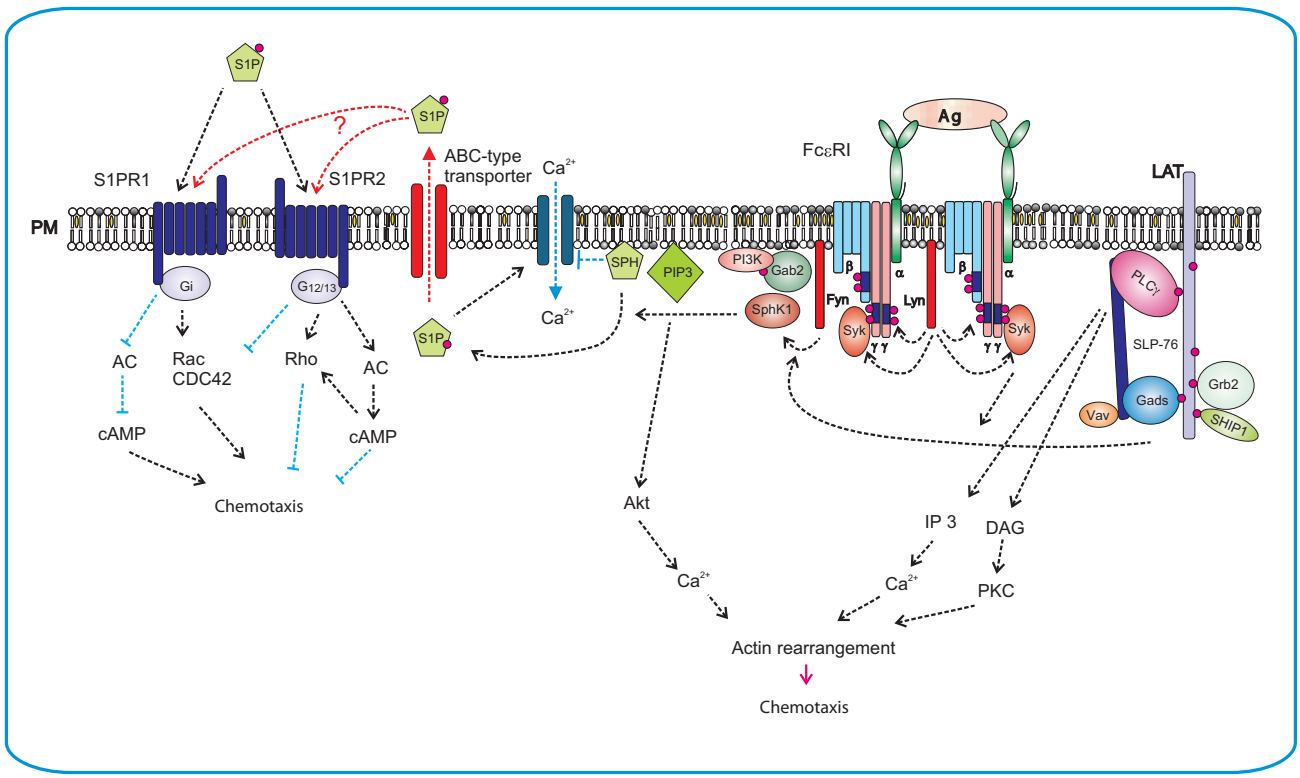

FIGURE 3 | Production of S1P after FcεRI triggering and S1P-mediated chemotaxis. FcεRI triggering leads to rapid activation of Lyn and Fyn kinases and Lyn-mediated phosphorylation of tyrosine residues of ITAM motifs in FcεRI β and γ subunits. This is followed by anchoring of Syk to FcεRI and enhanced enzymatic activity of Syk. Lyn kinase interacts directly with SphK1 and recruits it to the proximity of FcεRI, whereas Fyn kinase mediates the recruiting of Gab2 and PI3K to the membrane and foster in this way the activation of SphK1. Phosphorylation of sphingosine (SPH) by SphK1 leads to S1P formation. Sphingosine is an inhibitor of SOC channels. Formation of S1P results therefore in the release of SOC channels block. S1P can be transported from cytoplasm to the extracellular space by ABC-type transporter. Extracellular S1P then serves as a potent chemoattractant and promotes inflammation by recruiting other immune cells. Alternatively, S1P can affect mast cell activation and/or chemotaxis via two independent GPCRs, the S1PR1 and the S1PR2. S1PR1 acts via G_i subunit and induces mast cell migration by inhibiting of adenylate cyclase (AC) and activation of small GTPases Rac and Cdc42. S1PR2 is coupled to $G_{12/13}$ subunits that activate adenylate cyclase and thereby inhibit chemotaxis.

of SphK1 activity by interacting directly with SphK1; this interaction facilitates the recruitment of this lipid kinase to FcεRI (Urtz et al., 2004). In contrast, Fyn was found obligatory for activation of both SphKs. Fyn phosphorylates Gab2, which then binds the p85 regulatory subunit of PI3K (Parravicini et al., 2002). Both Gab2 and PI3K activities were essential for SphK1 activation, and were partially responsible for SphK2 activation. This indicates that additional Fyn-dependent, PI3K-independent signals are required for activation of SphK2 (Olivera et al., 2006).

As already mentioned, S1P can mediate mast cell functions through its intracellular and extracellular activities. The intracellular targets of S1P have not yet been fully identified, but a direct correlation was found out between S1P production and uptake of Ca^{2+}. Sphingosine and its structural analogs act as specific inhibitors of SOC influx. Thus, one possible mechanism where by SphK2 might exert its activating effect on calcium influx could be mediated through reduction of the level of sphingosine by its phosphorylation, derepressing in this way the blocking of the SOC channels (Mathes et al., 1998). S1P released from the cells can function as a ligand for a family of five G-protein-coupled S1P receptors (S1PR) 1–5. Evident diversity in the expression pattern of S1PRs can be seen in cells of the immune system. S1PR1 is expressed by most immune cells, whereas other receptors (S1PR2–5) have a more limited distribution. All five receptors were found to be expressed in dendritic cells, whereas mast cells and macrophages express only S1PR1 and S1PR2; eosinophiles express S1PR1–3, T cells S1PR1 and S1PR4, B-cells S1PR1 and S1PR3, and NK cells S1PR5 (Rivera et al., 2008). S1PRs expressed on mast cells can react with S1P by paracrine/autocrine mechanism. S1P levels in tissues increase under inflammatory conditions. Extracellular S1P has chemotactic activity and can attract mast cells to sites of inflammation. Furthermore, S1P produced by mast cells can induce their degranulation and cytokine production. S1PRs, like other GPCRs, transduce signals by associating with G-proteins. S1PR1 interacts with G_i, which can modulate different pathways leading to cell survival through the PI3K/Akt pathway, cell migration through the PI3K and Rac pathways, and cell proliferation through the Erk pathway. In contrast, S1PR2 is coupled to G_i, G_q, and $G_{12/13}$; the latter two are responsible for degranulation. Experiments with knock-out or knock-down of S1PR1 and/or S1PR2 indicated that these receptors have a non-overlapping function in mast cells. Loss of S1PR1 resulted in decreased chemotactic motility, whereas loss of S1PR2 inhibited mast cell degranulation. Because of the fact that concentrations of S1P required for degranulation were higher than those required for chemotaxis (Jolly et al., 2004) and higher concentrations of S1P also inhibited chemotaxis (Jolly et al., 2005), it has been suggested that mast cells in allergic inflammatory reactions would migrate to target tissues at low S1P concentration gradients. However, after reaching the target, higher S1P concentrations would prevent further migration and the cells could start to degranulate in response to a more extensive ligation of S1PRs (Olivera and Rivera, 2005).

EICOSANOIDS

Upon activation with specific antigen, complement or other transmembrane stimuli, mast cells produce several eicosanoids through activation of cytoplasmic phospholipase A2 (cPLA2), which releases free arachidonic acid from the plasma membrane (**Figure 4**). Free arachidonic acid can be metabolized to eicosanoids by three distinct pathways. One depends on the activity of lipoxygenases (LOXs) and leads to production of leukotrienes such as LTB_4, LTC_4, and LTD_4. The second is dependent on cyclooxygenases (COXs), which are engaged in the mechanisms responsible for production of prostaglandins such as PGE_2 and PGD_2. The third includes the cytochrome P450 monooxygenase that metabolizes arachidonic acid into epoxyeicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), and hydroperoxyeicosatetraenoic acids (HPETEs; Boyce, 2007; Wang and Dubois, 2010).

As indicated above, the key enzyme in initial stages of these processes is cPLA2. It can be activated by two major routes. One is Erk dependent and results in phosphorylation of the enzyme (Berenbaum et al., 2003), and the other appears to be Erk independent but rides on PI3K/Btk/PLCγ pathway enabling Ca^{2+}-dependent translocation of cPLA2 to the membrane (Clark et al., 1991; Kramer and Sharp, 1995, 1997).

Eicosanoids can act as proinflammatory mediators through specific receptors and serve as chemoattractants for various cell types including mast cells and their progenitors. The balance between self-secreted mediators and those produced by other cells is responsible for the paracrine versus autocrine regulation of mast cells (Boyce, 2007). More information on these mediators and their effects on mast cells is given below.

Prostaglandin E2

Eicosanoid PGE_2 is synthesized and secreted in response to inflammatory stimuli by many cell types, including dendritic cells, epithelial cells, fibroblasts, macrophages, and mast cells. Physiological functions of PGE_2 are mediated by four types of GPCRs, EP1–4. Each of these receptors is unique in tissue distribution, pharmacology, and action mechanism. The EP1 receptor is coupled to PLC and its stimulation leads to the increase in intracellular Ca^{2+}. Engagement of EP2 and EP4, induces an increase in cAMP level via G-protein α-subunit stimulation of adenylate cyclase. EP3 receptor exists in several isoforms generated by alternative splicing; these isoforms are coupled with different G-proteins (G_i, G_q, and G_s). The major signaling pathways described for EP3 receptor lead to a decrease in intracellular cAMP levels and increase in intracellular Ca^{2+}(Ichikawa et al., 1996; Kuehn and Gilfillan, 2007). In FcεRI-triggered cells, PGE_2 regulates activation in positive or negative way, depending on the EP receptors engaged (**Figure 5**). Enhancement of FcεRI-mediated degranulation and cytokine production was described for mouse BMMCs activated by PGE_2 bound to EP3 receptor (Gomi et al., 2000; Nataraj et al., 2001; Nguyen et al., 2000) or for human mast cells with PGE_2 bound to EP1 or EP3 (Wang and Lau, 2006). Another study reported inhibition of histamine, eicosanoids, and TNF-α production in human mast cells triggered by binding of PGE_2 to EP2 receptor, followed by inhibition of elevated cAMP levels (Kay et al., 2006). Recently, PGE_2 was described to act as a chemotactic factor for immature as well as mature murine mast cells *in vitro* (Weller et al., 2007). The same study described accumulation of mast cells *in vivo* after i.d. injection of PGE_2 (Weller et al., 2007). It was also shown that PGE_2 mediated chemotactic response in human mast cells (Kuehn

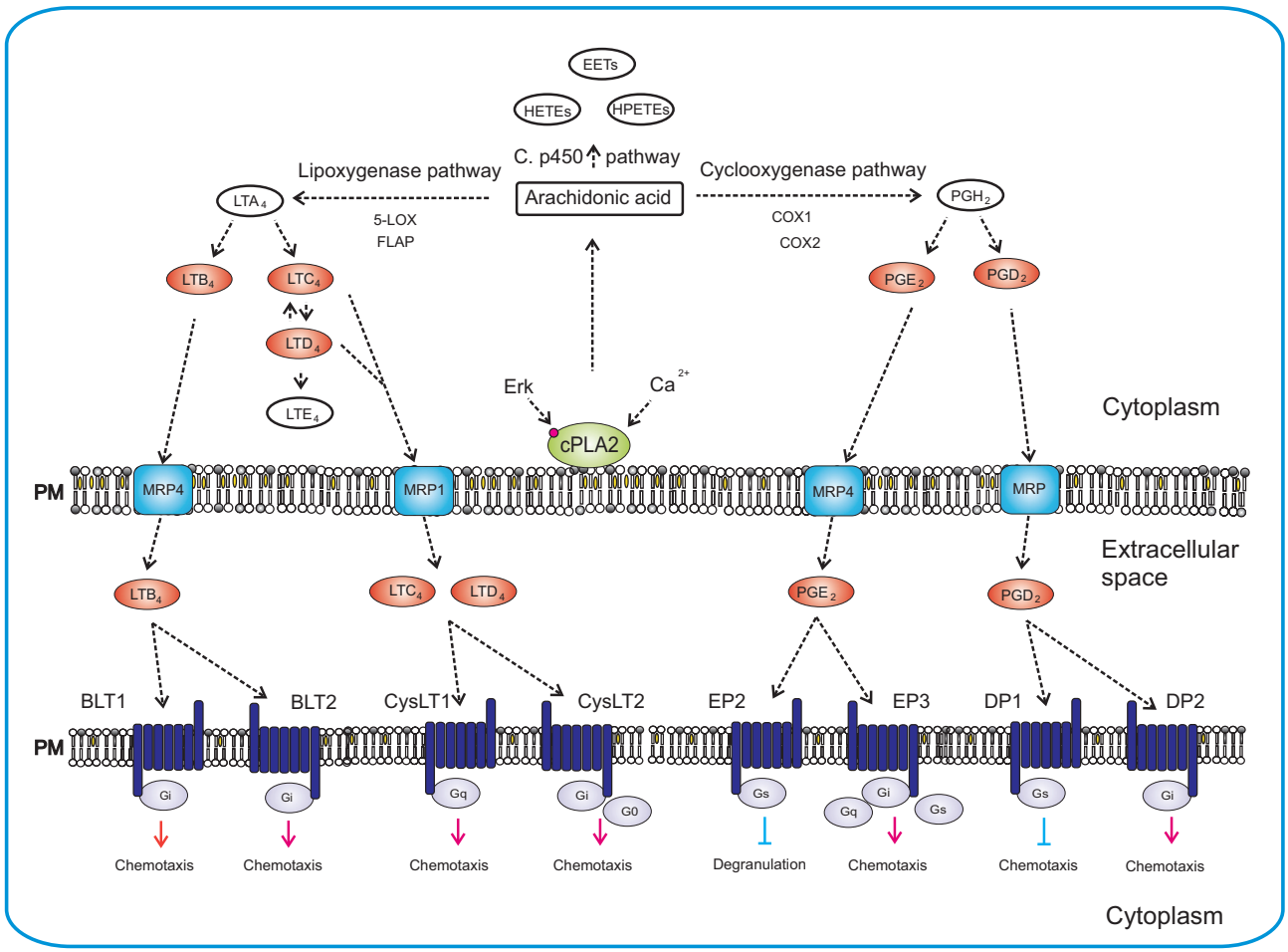

FIGURE 4 | Synthesis of eicosanoids and their actions in mast cells. Synthesis of eicosanoids is initiated by activation of cPLA2 via Erk-dependent pathway, followed by Ca^{2+}-dependent translocation of cPLA2 to the plasma membrane where it liberates the arachidonic acid. Free arachidonic acid can be metabolized by three independent pathways: lipoxygenase pathway, cyclooxygenase pathway and cytochrome P450 monooxygenase pathway. The 5-lipoxygenase in cooperation with FLAP mediates transition of arachidonic acid to unstable LTA_4. LTA_4 is subsequently converted to LTB_4, LTC_4, and LTD_4. In the cyclooxygenase pathway, arachidonic acid is converted to intermediate PGH_2 by peroxidase activity of COX enzymes. PGH_2 is then metabolized by specific enzymes to final products that include PGE_2 and PGD_2. Cytochrome P450 metabolizes arachidonic acid to epoxyeicosatrienoic acids (EETs), hydroperoxyeicosatetraenoic acids (HPETEs), and hydroxyeicosatetraenoic acids (HETEs). The final products are transported outside the cell by specific multidrug resistance protein (MRP) ABC-type transporters and can act there as chemoattractants for mast cells, their progenitors or other cells. Eicosanoids mediate their action via different GPCRs (BLT1, BLT2, CysLT1, CysLT2, EP2, EP3, DP1, and DP2) localized on the plasma membrane of target cells. Eicosanoids in red bodies are those secreted from mast cells and acting as chemoattractants on various targets.

et al., 2011a). Biochemical studies showed that PGE_2 mediated chemotaxis in mast cells through engagement of GPCRs linked to EP3 receptor. Unlike the FcεRI or c-Kit, GPCRs do not require the engagement and activation of tyrosine kinases and protein tyrosine phosphorylation for initial propagation of the signals but reckon instead on coupling to a heterotrimetric complex of G_α and $G_{\beta\gamma}$ subunits (Dorsam and Gutkind, 2007). PGE_2 activation of the EP3 receptor initiated mobilization of PI3K and Akt, but this pathway in contrast to antigen-mediated triggering, was independent of Btk. Other targets involved in PGE_2-induced chemotaxis, downstream of PI3K, are still vaguely understood. Two forms of mTOR, the mTORC1 and mTORC2, have recently been found to be activated downstream of PI3K in BMMCs and have therefore been suspected to play an important role in PGE_2-mediated chemotaxis. However, pharmacological studies with specific inhibitors of mTORCs as well as siRNA knock-down experiments showed that only mTORC2 cascade was selectively utilized in chemotaxis toward PGE_2. EP3-induced chemotaxis thus differs from c-Kit-mediated chemotaxis, which is partly controlled also by mTORC1 (Kim et al., 2008a). Rac-dependent actin reorganization has also been observed in PGE_2-mediated chemotaxis but it makes use of a different pathway independent of mTORC2 (Kuehn et al., 2011b).

Prostaglandin D2

Eicosanoid PGD_2 is another arachidonic acid metabolite with important roles in various inflammatory diseases. It is produced by IgE-activated mast cells through the COX pathway (Lewis et al.,

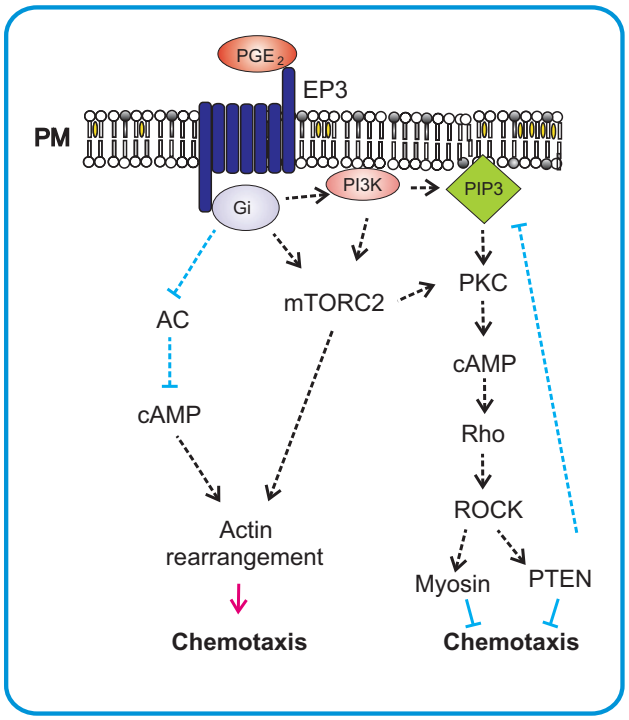

FIGURE 5 | PGE_2-mediated chemotaxis. PGE_2 mediates its chemotactic action on mast cells via Gi-coupled EP3 receptor. After activation the α-subunit of G-protein inhibits adenylate cyclase (AC) and thereby causes a decrease of cAMP which leads to stimulation of migration. This stimulation also results in activation of mTORC2, an important regulator of actin rearrangements and positive regulator of chemotaxis. mTORC2 can also influence PKC-dependent activation of small GTPase Rho and subsequent activation of PTEN through ROCK. This can act as a negative feedback for production of PIP3 by PI3K that is also activated by PGE_2 in mast cells.

1982). PGD_2 was found to bind to two different prostaglandin receptors, G_s-coupled DP1 that stimulates adenylate cyclase, and DP2 (also known as chemoattractant receptor-homologous molecule expressed on Th2 cells, CRTH2) that is coupled to G_i. These two membrane-bound receptors show extensively antagonistic effects. PDG_2 was found to inhibit migration of several cell types such as dendritic cells and Langerhans cells through the DP1 receptor (Hammad et al., 2003; Angeli et al., 2004; Yamamoto et al., 2011), and to stimulate migration of lymphocytes, eosinophils, and basophils via the DP2 receptor (Hirai et al., 2001; Shiraishi et al., 2005; Yamamoto et al., 2011). The DP2-dependent migration has recently been reported also for mast cells (Boehme et al., 2009).

Leukotriene B4

Eicosanoid LTB_4, as a potent activator and chemoattractant for leukocytes, is implicated in various inflammatory diseases. It is produced from arachidonic acid via the 5-LOX pathway by innate immune cells such as neutrophils, macrophages, and mast cells (Toda et al., 2002; Osher et al., 2006; Ohnishi et al., 2008). The action of LTB_4 is mediated by two GPCRs, the BLT1 and BLT2. BLT1, a high affinity receptor for LTB_4, is expressed predominantly in leukocytes including granulocytes, monocytes, macrophages, dendritic cells, effector T cells, and mast cells (Yokomizo et al., 1997; Yokomizo et al., 2000), whereas BLT2, a low affinity receptor which can also bind to some other eicosanoids is expressed ubiquitously (Yokomizo et al., 2000). Both BLT1 and BLT2 couple to the inhibitory G_i protein, inhibit adenylate cyclase, activate PLCγ to increase intracellular Ca^{2+}, and induce robust chemotaxis (Yokomizo, 2011). Mouse BLT2 is highly expressed in small intestine and skin, and BLT2-selective agonist induces chemotaxis in primary mouse keratinocytes and BMMCs. Triggering of BMMCs by LTB_4 induces transient Erk and Akt phosphorylation without any changes of p38 phosphorylation (Lundeen et al., 2006).

LTB_4 serves as a potent chemoattractant for mast cell progenitors, but not mature mast cells (Weller et al., 2005). It was also shown that BLT2 is upregulated in cells activated via FcεRI. Knock-down of BLT2 resulted in reduced NOX1-Ros-mediated production of Th2 cytokines such as IL-4 and IL-13 (Cho et al., 2010). On the other hand, exposure of BMMCs to SCF, another chemoattractant and activator, resulted in downregulation of BLT1 and BLT2 expression and inhibition of migration toward LTB_4 (Lundeen et al., 2006).

Leukotriene D4 and Leukotriene C4

Similarly to LTB_4, the LTD_4, and LTC_4 are produced in activated mast cells via the LOX pathway (Razin et al., 1983). These two leukotrienes act through two specific receptors CysLT1 and CysLT2 that have non-redundant functions in mast cells and are coupled to distinct intracellular signaling pathways. CysLT1 receptor is bound to G_q proteins, whereas CysLT2 receptor uses G_i/G_o proteins for calcium-independent signaling (Bautz et al., 2001; Möhle et al., 2001; Mellor et al., 2003). Both LTD_4 and LTC_4 were found to function as co-mitogens for low concentrations of SCF (Jiang et al., 2006, 2007). LTD4 induces migration and transendothelial migration of $CD34^+$mast cell progenitors (Bautz et al., 2001).

CHEMOKINES

Chemokines are members of a superfamily of 8–15 kDa heparin-binding chemotactic cytokines that function as potent chemoattractants for different cell types in the immune system. Although they are not homologous sequentially, they share a similar three-dimensional structure. There are four subclasses of chemokines classified according to the location of the first two cysteines in their sequence: CC, CXC, C, and CX3C (where X stands for any amino acid). Major families, the CC and CXC, have more than 50 members, whereas C and CX3C families are much smaller with only one or a few members per family. Some chemokines are expressed constitutively by many cell types in tissue-specific manner. Expression of other chemokinesis induced only under specific conditions, typically in response to inflammatory signals (Kaplan, 2001; Sabroe et al., 2002; Juremalm and Nilsson, 2005; Scott and Bradding, 2005; Lloyd and Brown, 2006; Hallgren and Gurish, 2011). Chemokine receptors belong to GPCRs, and are mostly capable of binding more than one chemokine. Considerable differences in the expression of chemokine receptors exist between mature and immature mast cells and among individual mast cell

subtypes. For example, mast cells derived from progenitors present in human cord blood express several chemokine receptors including CCR3 (eotaxin receptor), CCR5 [receptor for the Th1-active chemokine macrophage inflammatory protein (MIP)-1α], CXCR2 (IL-8 receptor), and CXCR4 [receptor for the constitutively produced lymphocyte chemoattractant stromal cell-derived factor (SDF)-1α]. Only the CCR3 was retained on mature human mast cells (Ochi et al., 1999; Bautz et al., 2001).

Chemokines exhibit a different pattern of expression depending on mast cell type. For example, CCR3, CXCR1, CXCR3, and CXCR4 were found to be highly expressed by human LMCs, and their respective ligands, i.e., CCL11 (eotaxin), CXCL8 (IL-8), CXCL10, interferon γ-induced protein (IP)-10, and CXCL12 (SDF-1α), mediated LMC chemotaxis (Brightling et al., 2005a,b). Interestingly, the CXCR3 was missing among those reported to be expressed on human BMMCs. Cord blood-derived mast cells and LMCs from patients with allergic asthma expressed CCR1 and CCR4 (Juremalm et al., 2000, 2002; Amin et al., 2005) and CCR3 was expressed in human skin and intestinal mast cells (Romagnani et al., 1999). Recent work testing the expression of chemokines and chemokine receptors showed that human intestinal mast cells expressed nine chemokine receptors (CCR6–8, CXCR1–4, XCR1, and CX3CR1) and 27 chemokines. Whereas expression of chemokine receptors was independent of antigen stimulation, the expression of 12 chemokines (CCL1–CCL5, CCL7, CCL18, CCL20, CXCL2, CXCL3, CXCL8, and XCL1) was more than fourfold upregulated upon antigen triggering (Feuser et al., 2012).

Mouse mast cells also express several chemokine receptors. Studies with mouse BMMCs showed expression of mRNA for CCR1–5 and its upregulation after activation through c-kit or FcεRI (Oliveira and Lukacs, 2001). Chemotaxis assays showed that mast cells migrated toward CCL2 and CCL5. If IgE-sensitized cells were exposed to antigen, enhanced migration was also observed toward CXCL4 and CCL3 (Taub et al., 1995). In another study mouse BMMCs expressed CCR3 mRNA, but the corresponding protein was not detected and migration toward CCR3 ligands (CCL11 and CCL24) was noticed neither in mature nor immature cells. In addition, no phenotypic differences between WT and $CCR3^{-/-}$ mice were found (Collington et al., 2010b, 2011). The observed differences could be attributed to different mouse strains chosen or different methods used for growth of the cells. The first group (Oliveira and Lukacs, 2001) cultured BMMCs in the presence of IL-3 and SCF (both at a concentration of 15 ng/ml), whereas in the second study only IL-3 at 5 ng/ml was used. The data also suggest that the role of CCR3 in chemotaxis depends not only on cell origin (mouse versus human) but also on physiological settings. Expression of different chemokines could be responsible for mast cell homing into different tissue. Impaired homing was found in mouse mast cell progenitors lacking genes for selected chemokine receptors. When $CXCR2^{-/-}$ cells were used, mast cells were absent in intestine but still present in lung, spleen, or bone marrow (Abonia et al., 2005). Homing to intestines was unaltered in mice lacking CCR2, CCR3, and CCR5. On the other hand, knock-out of CCR3 led to an increased number of intratracheal mast cells (Humbles et al., 2002; Hallgren and Gurish, 2007; Hallgren et al., 2007). Immature BMMCs, when cultured in the presence of IL-3 and SCF but not IL-3 alone, migrated in response to CCL2. Recruitment of mast cell progenitors to the allergen-sensitized lungs was significantly reduced in both $CCR2^{-/-}$ and $CCL2^{-/-}$ mice. However, repopulation studies using sublethally irradiated and bone marrow reconstituted mice indicated that receptor and ligand expression was more important on stromal cells than mast cells (Collington et al., 2010a). Dermal mast cells were found to play a critical role in the induction of immune suppression. UV exposure caused increased migration of mast cells from skin to the draining lymph nodes. This was mediated by interaction of CXCR4 expressed on mast cells with CXCL12 that is up regulated in lymph node B-cells (Byrne et al., 2008).

Murine BMMCs of both connective tissue and mucosal phenotype were also found to express CX3CR1 and were attracted by its chemoattractant CX3CL1 (fractalkine), which is a membrane-bound peptide. Fractalkine was unable to induce degranulation quantified by the release of granule-associated β-hexosaminidase (Papadopoulos et al., 2000).

TRANSFORMING GROWTH FACTOR β

Multifunctional transforming growth factor (TGF)-β is a peptide that plays a key role in tissue cell growth, differentiation, morphogenesis, and formation of extracellular matrix, as well as in coordination of the complex events leading to tissue repair after injury. Its pivotal function within the immune system is to maintain tolerance by regulating the lymphocyte proliferation, differentiation, and survival. Furthermore, TGF-β controls the initiation and resolution of inflammatory responses through the regulation of chemotaxis and activation of lymphocytes, dendritic cells, natural killer cells, granulocytes, and mast cells. Through its pleiotropic effect on various immune cells, TGF-β prevents the development of autoimmune diseases without compromising the efficiency of immune responses toward pathogens (Wrzesinski et al., 2007). Initial studies on chemotaxis showed that TGF-β at femtomolar concentrations caused directed migration of cultured mouse cells, with maximal chemotactic response observed at 25 fM. In its ability to induce directed migration, TGF-β was substantially more efficient on the molar basis than other mast cell chemoattractants, including SCF (Gruber et al., 1994). Detailed studies showed that TGF-β3, one of the three isoforms of TGF-β, was more effective than TGF-β1 and TGF-β2 when tested at a constant concentration of 40 fM. The effect was observed in both human mast cell line HMC-1 and cultured primary mast cells. Furthermore, TGF-β1, TGF-β2, and less efficiently TGF-β3 inhibited the proliferation of HMC-1 cells. The migratory responses were probably mediated through interaction of all TGF-β isoforms with TGF-β serine/threonine type I and II receptors that were found expressed in HMC-1 cells (Olsson et al., 2000). Application of peptidoglycan from *Staphylococcus aureus* to barrier-disrupted abdominal skin lead to accumulation of mast cells at the site of application. This accumulation was abrogated by administration of TGF-β1 Ab. These results suggest that peptidoglycan have ability to induce mast cell accumulation through TGF-β1 production by epidermal keratinocytes (Matsui and Nishikawa, 2005). All combined, the data indicate that TGF-β isoforms are highly potent chemoattractants for mast cells and could possibly play an important role in the recruitment of mast cells to the sites of inflammatory reactions.

ADENOSINE

The purine nucleoside adenosine is produced by numerous cell types in response to cell stress and hypoxia. It binds to four types of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3. The latter three were found expressed on mast cells (Ramkumar et al., 1993; Ralevic and Burnstock, 1998). These receptors are GPCRs and are coupled to different G-proteins: A_{2A} receptor to G_s, A_{2B} to G_s or G_q and A_3 to G_i or G_q (Kuehn and Gilfillan, 2007). Adenosine regulates mast cell activities positively or negatively, depending on the plasma membrane receptor involved and mast cell type (Kuehn and Gilfillan, 2007; Brown et al., 2008). It has been reported that adenosine regulates FcεRI-mediated mast cell degranulation, cytokine production, and chemotaxis. Binding of adenosine to A_{2A} receptor also blocked the potassium channel, KCa3.1, and inhibited migration of human LMCs in response to asthmatic airway smooth muscle conditioned medium (Duffy et al., 2007). Adenosine alone was unable to cause migration of BMMCs but it potentiated the migration toward antigen in a pertussis-toxin dependent manner. This points to A_3 receptor coupled to G_i (Kuehn et al., 2010). It may possible be relevant that purine and pyrimidine nucleotides ADP, ATP, and UTP, acting at low micromolar concentrations, also caused directed migration of rat BMMCs (McCloskey et al., 1999).

Other chemoattractants

There are several other molecules that are recognized by mast cells as chemoattractants. Human C1q is a collagen-like glycoprotein that associates with the Ca^{2+}-dependent Clr_2Cls_2 tetramer, forming the first component of complement, C1. C1q induces mast cell migration in a specific and dose-dependent manner. Two specific receptors were found on murine and human mast cells, the cC1q-R that binds to the collagen-like stalk of Clq, and the gC1q-R that binds to the globular "heads" of C1q (Ghebrehiwet et al., 1995).

C3a and C5a are other complement components which are potent chemoattractants for eosinophils and neutrophils but in addition can attract mast cells and basophils. The chemotactic response is blocked by pertussis-toxin, suggesting that G_i-coupled receptors are involved in signal transduction (Nilsson et al., 1996; Hartmann et al., 1997).

Platelet-activating factor (PAF) is a potent phospholipid inflammatory mediator that is released from a variety of cells, including endothelial cells, neutrophils, and macrophages. PAF acts via binding to a specific GPCR. PAF was also found to induce mast cell migration in pertussis-toxin dependent manner this indicates that G_i-coupled receptor is involved in this process (Nilsson et al., 2000).

Another chemoattractant, the 5-hydroxytryptamine (5-HT), is implicated in enhancing inflammatory reactions of skin, lung, and gastrointestinal tract. It was reported that both human and mouse mast cells expressed a variety of 5-HT GPCRs, but the predominant receptor mediating the effects like adhesion and migration on these cells was the 5-HT1A G_i-coupled receptor. Mast cells also synthesize 5-HT, which can function in both paracrine and autocrine manner (Kushnir-Sukhov et al., 2006).

Recently published studies also showed that the neuroendocrine peptide catestatin, as well as its naturally occurring variants were capable of inducing migration, degranulation, and production of cytokines, eicosanoids, and chemokines, in both human mast cell line LAD-2 and peripheral blood-derived mast cells (Aung et al., 2011).

Furthermore, proinflammatory cytokines were capable of inducing chemotaxis. Mast cells were shown to migrate in response to IL-6 or TNF-α stimulation. IL-6-stimulated mast cell migration was the result of chemokinesis, whereas TNF-α-induced migration was based on chemotaxis (Brzezińska-Błaszczyk et al., 2007; Misiak-Tloczek and Brzezińska-Blaszczyk, 2009). In contrast, IL-10 inhibited mast cell migration toward RANTES, TNF-α, and nerve growth factor (Pietrzak et al., 2011).

Mast cells were also found to accumulate in proximity of various malignant tumors. It has been shown that tumor-derived tripeptides glycylhistidyllysine and *N*-formyl-methionyl-leucyl-phenylalanine are potent chemoattractants for rat peritoneal mast cells, suggesting that they could be important in early events of tumor neovascularization (Poole and Zetter, 1983). Adrenomedullin is another peptide amid produced by human cancer cells that was found to be chemotactic for HMC-1 (Zudaire et al., 2006).

LESSONS FROM OTHER CELLS

There exist two important cellular models for studying chemotaxis of eukaryotic cells, neutrophils, and *Dictyostelium*. Both can detect very shallow differences in the surrounding gradients of chemoattractants and are thereby capable of quickly changing directions of their movement. Because GPCRs that have often been the subjects of studied on chemotaxis of the two cell types are also involved in migration of mast cells, the knowledge obtained in studies on neutrophils and *Dictyostelium* can be helpful for better understanding of chemotaxis in mast cells. In migrating cells, two sides can be distinguished, the front side (also called leading edge) and the rear side. Concentration of chemoattractants reaching the cell surface is, consequently highest in the front and lowest in the rear. In both, neutrophils and *Dictyostelium*, one of the first processes occurring in the leading edge after sensing chemoattractant is activation of PI3K resulting in accumulation of PIP3 in the leading edge membrane (Nilsson et al., 1996; Hartmann et al., 1997; Funamoto et al., 2001, 2002; Huang et al., 2003). PIP3 than serves as a binding site for a diversity of pleckstrin homology (PH)-domain containing proteins. The most important proteins recruited appear to be guanine nucleotide exchange factors (GEFs) for Rac GTPases, such as dedicators of cytokinesis (DOCKs). Their recruitment leads to activation of small GTPase Rac and subsequently localized polymerization of F-actin. The rear side of neutrophils is engaged in activation of another small GTPase RhoA that activates ROCK and makes thus possible the phosphorylation of myosin light chain. These signals are mediated by different G-proteins. At the leading edge, it is mediated by a G_i-coupled protein which inhibits adenylate cyclase, whereas $G_{12/13}$ promote back signal by myosin II. Despite the fact that generating PIP3 is crucial for cell migration, inhibition of PIP3 formation in neutrophils (Ferguson et al., 2007; Heit et al., 2008), or in *Dictyostelium* (Loovers et al., 2006; Andrew and Insall, 2007) did not, at least under certain circumstances, fully inhibited cell migration, but only slowed it down. The data suggest that PIP3 accumulation is not essential for direction of chemotaxis but controls the extent of random generation of pseudopodia. The steep PIP3 gradient is also controlled by

degradation of PIP3 by phosphatases, mainly at the rear side of the migrating cell. PTEN was found to be a dominant phosphatase for PIP3 degradation in *Dictyostelium* (Funamoto et al., 2002; Iijima et al., 2002, 2004). In contrast, PTEN played only a minor role in chemotaxis of neutrophils (Subramanian et al., 2007) where SHIP1 was the major regulator (Nishio et al., 2007). This finding was supported by experiments indicating that neutrophils lacking SHIP1 exhibited severe defects in neutrophil polarization and motility. In macrophages, PTEN was found to control PI3K activity via negative feedback including the PI3K/Rho/ROCK pathway (Papakonstanti et al., 2007). These results show that several alternative pathways are involved in chemotaxis. One of them could involve another lipid signaling pathway parallel to PI3K and mediated through phospholipase A2 (PLA2; Chen et al., 2007). Each of these pathways can inhibit chemotaxis in shallow gradients, whereas inhibition of chemotaxis in steep gradients requires both pathways to be disrupted (Kolsch et al., 2008; Wang et al., 2011).

The behavior of cells in different concentration gradients is diverse. In steep gradients, neutrophils and *Dictyostelium* can project pseudopodia directly up the gradient and move toward the chemoattractant source with only little deviation. In shallower gradients, cells produce daughter pseudopods and then choose the one that leads up to the highest concentration of chemoattractant. However, in the weakest gradients the movement of neutrophils and *Dictyostelium* looks like a biased random walk (Kay et al., 2008; Insall, 2010). Key regulators of cellular polarity in migrating neutrophils and *Dictyostelium* are mTORC2 (Liu et al., 2010) and TORC2 (Lee et al., 2005). Neutrophils lacking mTORC2 activity had strongly reduced chemotactic ability, and were unable to polarize. Actin polymerization and myosin II regulation were impaired independently. Also production of cAMP was affected. The effect of mTORC2 on myosin II filament assembly was mediated via cAMP, RhoA, and ROCK-dependent way (Charest and Firtel, 2010; Charest et al., 2010; Liu et al., 2010). Myosin phosphorylation stimulates its activity but the stability of myosin filaments is regulated by its dephosphorylation. Based on these and other studies it was concluded that activation of the receptors triggers both promoting and inhibiting signals for myosin phosphorylation and dephosphorylation, and this cycling is important for optimal migration (Liu et al., 2010). A recent study with *Dictyostelium* mutants focused on better understanding of the roles of PI3K, TORC2, PLA2, and soluble guanylate cyclase in chemotaxis in gradients of different strength. The study showed that Ras activation at the leading edge was a basal signaling module sufficient for chemotaxis in this system. The enzymes under study were not required for Ras activation in steep gradients of cAMP but were important for the direction and improved orientation in shallow cAMP gradients (Kortholt et al., 2011).

CONCLUDING REMARKS

Mast cells are known for their role in allergy and as effector cells of innate immunity. They are distributed everywhere in the body and are enriched at boundaries of the body, such as respiratory and gastrointestinal mucosa and skin. They are also found near blood vessels and nerve endings where they contribute to protection against various pathogens. They must be recruitable to all these sites by precise cumulation mechanisms to warrant equal distribution without any crowding or insufficient representation in some places. This would explain why mast cells have so many surface receptors involved in chemotaxis. On the other hand, an important homeostatic role of a crosstalk between mast cells and other immune and non-immune cells and its enhancement after mast cell triggering could explain why mast cells produce so many chemoattractants and why this production is enhanced after mast cell activation. Furthermore, mast cells of human and mouse origin also differ in the expression of chemoattractant receptors and chemoattractants produced (**Table 1**).

Although numerous molecules have been identified as significant players in chemotaxis, it is very likely that other molecules involved in chemotaxis will be discovered. Some controversial results mentioned in this review are attributable to different mast cell types used, various methods used for detection of chemotaxis or some additional factors like culture conditions, number of passages of *in vitro* cultured cells before the cells are used, origin of mast cells, age and genotype of donors and others. The crosstalk between different receptors and signaling molecules is still an open question, which remains also to be solved. Important data reflecting cellular potential and commitment can be expected from analysis of whole genome transcriptome of the cells under study and from application of methods of systems biology in studies of mast cell chemotaxis under *in vitro* and *in vivo* conditions.

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Cytoskeleton in mast cell signaling

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Mast cell activation mediated by the high affinity receptor for IgE (FcεRI) is a key event in allergic response and inflammation. Other receptors on mast cells, as c-Kit for stem cell factor and G protein-coupled receptors (GPCRs) synergistically enhance the FcεRI-mediated release of inflammatory mediators. Activation of various signaling pathways in mast cells results in changes in cell morphology, adhesion to substrate, exocytosis, and migration. Reorganization of cytoskeleton is pivotal in all these processes. Cytoskeletal proteins also play an important role in initial stages of FcεRI and other surface receptors induced triggering. Highly dynamic microtubules formed by αβ-tubulin dimers as well as microfilaments build up from polymerized actin are affected in activated cells by kinases/phosphatases, Rho GTPases and changes in concentration of cytosolic Ca^{2+}. Also important are nucleation proteins; the γ-tubulin complexes in case of microtubules or Arp 2/3 complex with its nucleation promoting factors and formins in case of microfilaments. The dynamic nature of microtubules and microfilaments in activated cells depends on many associated/regulatory proteins. Changes in rigidity of activated mast cells reflect changes in intermediate filaments build up from vimentin. This review offers a critical appraisal of current knowledge on the role of cytoskeleton in mast cells signaling.

Keywords: actins, intermediate filaments, mast cell activation, microfilaments, microtubules, signal transduction, tubulins, vimentin

INTRODUCTION

Mast cells play an essential role in innate immunity, allergy, and inflammation. When activated they release mediators that are pivotal for initiation of inflammatory reactions associated with allergic disorders. Activation of mast cells in allergic inflammatory response occurs via the high affinity receptor for IgE (FcεRI) following receptor aggregation induced by antigen-mediated cross-linking of IgE occupied FcεRI. This response is profoundly influenced by other factors that modulate the threshold levels of mast cell triggering. Cell-surface receptors, such as the stem cell factor (SCF) receptor, c-Kit, and the specific G protein-coupled receptors (GPCRs) synergistically enhance FcεRI-mediated mast cell exocytosis (degranulation) (Kalesnikoff and Galli, 2008; Gilfillan and Rivera, 2009).

The first defined step in FcεRI signaling is phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the FcεRI β and γ subunits by Src-family protein tyrosine kinase (PTK) Lyn. Tyrosine-phosphorylated ITAMs of the FcεRI γ subunits serve as docking sites for the tandem Src homology (SH)2 domains of the non-receptor tyrosine kinase Syk (spleen tyrosine kinase) of the Syk/Zap-70 family. Syk bound to ITAMs adopts an active conformation that facilitates its phosphorylation by Lyn and further increase in enzymatic activity. Consequently, Syk phosphorylates a number of its targets critical for further propagation of the signal, including the transmembrane adaptor linker proteins (TRAPs), linker for activation of T cells (LAT), and non-T cell activation linker (NTAL) (Dráber et al., 2012). Phosphorylated TRAPs serve as plasma membrane docking sites for cytoplasmic SH2 domain containing molecules like growth factor receptor-bound protein 2 (Grb2) and phospholipase Cγ (PLCγ). After membrane anchoring and activation, PLCγ hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) and produces diacylglycerol (DAG) and inositol 1,4,5,-triphosphate (IP_3) that binds to its receptors on endoplasmic reticulum (ER). This results in Ca^{2+} efflux from ER. Subsequently, depletion of Ca^{2+} from ER lumen induces Ca^{2+} influx across the plasma membrane through store-operated Ca^{2+} channels. The influx leads to enhancement in free cytoplasmic Ca^{2+} concentration, a step which is substantial for further signaling events (Blank and Rivera, 2004; Rivera et al., 2008). The stimulatory effect of cross-linking of IgE receptors with antigen can be mimicked in part by pervanadate, a compound that effectively inhibits protein tyrosine phosphatases (Teshima et al., 1994).

Mast cells also express on their surfaces receptors for a number of ligands known to be potent mast cell chemoattractants. The most thoroughly investigated of these is SCF, a natural ligand for c-Kit (Meininger et al., 1992). Others include agonists of GPCRs, such as prostaglandin E2 (PGE_2), which positively regulates mast cell responses through the EP3 receptor (Weller et al., 2007). There is some evidence for the hypothesis that IgE sensitized mast cells migrate toward the antigen, potentially through the release of chemoattractants from the mast cells themselves (Ishizuka et al., 2001).

Degranulation can also be induced, independently of FcεRI aggregation, by thapsigargin, a compound that discharges intracellular Ca^{2+} stores by inhibition of the Ca^{2+}-ATPase pumps located in ER membrane (Thastrup et al., 1990). Similarly, stimulation with basic secretagogues, such as anaphylatoxins,

neuropeptides, compound 48/80, and poly-L-lysine, results in activation of distinct FcεRI-independent pathways (Lagunoff et al., 1983). These stimuli share a common characteristic of being polycations which are able to penetrate through the plasma membrane and stimulate the G-proteins (Bueb et al., 1992; Deng et al., 2009).

Activation of mast cells is accompanied by changes in cell morphology, enhanced adhesion to various substrates, migration, and exocytosis. All these processes are dependent on the activity of cytoskeletal proteins. The cytoskeleton of mast cells is formed by three types of filamentous structures: microtubules formed by tubulin dimers, microfilaments composed of actin, and intermediate filaments of vimentin-type. These cytoskeletal networks differ in their organization, protein compositon, and functions. In cells, there is equilibrium between soluble and polymeric state of principal building proteins that form corresponding cytoskeletal structures. Research has mostly focused on microfilaments and microtubules, that both play a critical role in these processes. Data on the involvement of intermediate filaments in mast cell activation are still very limited. A simplified model of early signaling events leading to cytoskeleton reorganization after FcεRI aggregation is shown in **Figure 1**.

Cytoskeleton of activated mast cells has been studied in various cellular models ranging from mouse bone marrow-derived mast cells (BMMCs) and isolated rat peritoneal mast cells (RPMCs) to mouse BMMC lines (e.g., MC/9), rat basophilic leukemia cell lines (e.g., RBL-2H3), or human mast cell lines (e.g., HMC-1). Typical distribution of microtubules and microfilaments in interphase RBL-2H3 cells is shown in **Figure 2**.

This review presents a critical survey of current knowledge on the role of cytoskeleton in mast cells signaling. Basic characteristic properties of particular cytoskeletal systems are outlined at the beginning followed by evaluation of their participation in mast cell activation.

MICROTUBULES

MICROTUBULES AND TUBULINS

Microtubules are cytoskeletal polymers essential for many cellular activities such as maintenance of cell shape, division, migration, positioning of organelles, and ordered vesicle transport powered

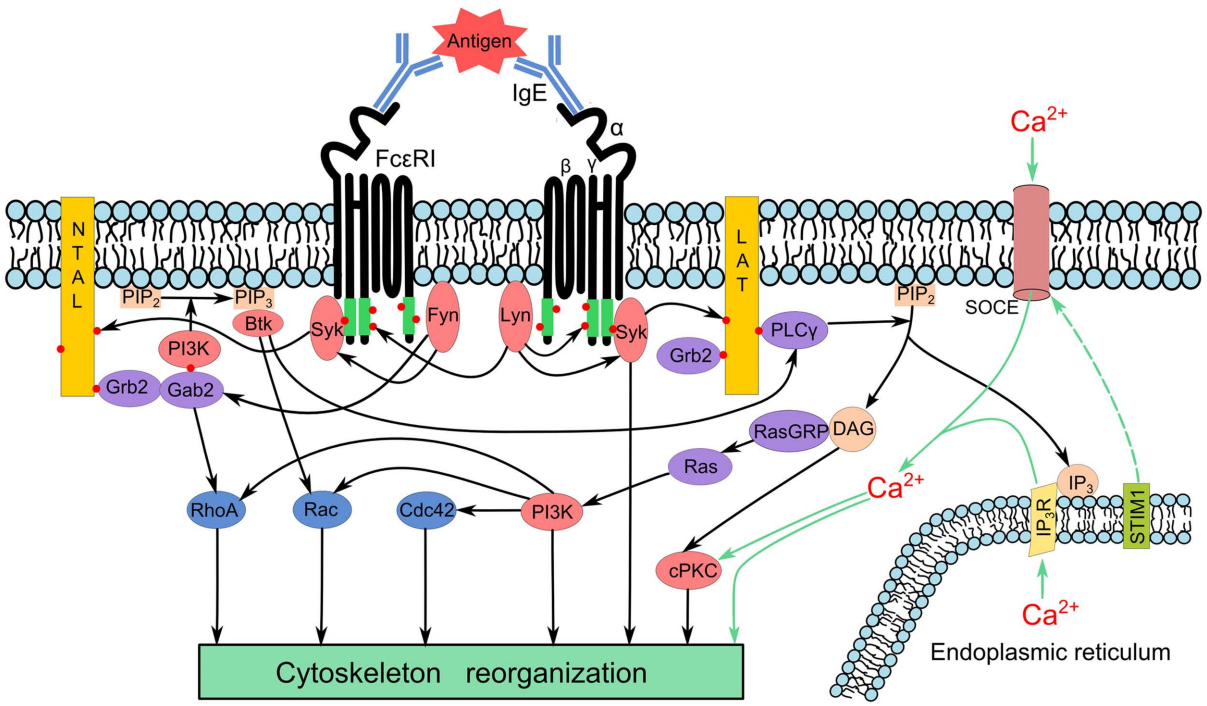

FIGURE 1 | Simplified model of early signaling events leading to cytoskeleton reorganization after FcεRI aggregation. Aggregation of the FcεRI by multivalent antigen induces activation of the Src kinase Lyn, which phosphorylates (red dots) ITAM motifs of FcεRI β and γ subunits (green), followed by anchoring of the Syk kinase to FcεRI through interaction of Syk-SH2 domains with phosphorylated ITAMs. Aggregation also leads to activation of Fyn kinase that phosphorylates the adaptor Gab2. Transmembrane adaptor proteins NTAL and LAT are phosphorylated by Syk giving rise to formation of binding sites for various SH2-containing proteins such as Grb2. In this way it brings Gab2 and phosphoinositide-3-kinase (PI3K) to the membrane. Activated PI3K phosphorylates phosphatidylinositol- 4,5-biphosphate (PIP_2) to yield phosphatidylinositol-3,4,5-triphosphate (PIP_3), a binding site for PH-domain containing proteins like kinase Btk. This process further propagates the signal through activation of phospholipase Cγ (PLCγ) that hydrolyses PIP_2 to inositol 1,4,5,-triphosphate (IP_3) and diacylglycerol (DAG). The binding of IP_3 to its receptor (IP_3R) triggers release of Ca^{2+} from endoplasmic reticulum; STIM1 couples depletion of Ca^{2+} from endoplasmic reticulum with activation of Ca^{2+} channels and influx of extracellular Ca^{2+} by the so called store-operated Ca^{2+} entry (SOCE). DAG and Ca^{2+} activate conventional protein kinase C (cPKC). DAG also recruits Ras guanyl nucleotide-releasing proteins (RasGRPs) for subsequent activation of Ras family proteins that stimulate PI3K. The PIP_3 also recruits nuclotide exchange factors that subsequently lead to activation of the Rho family of GTPases (RhoA, Rac, and Cdc42). Enhanced concentration of Ca^{2+} as well as activated kinases and GTPases modulate cytoskeleton arrangements.

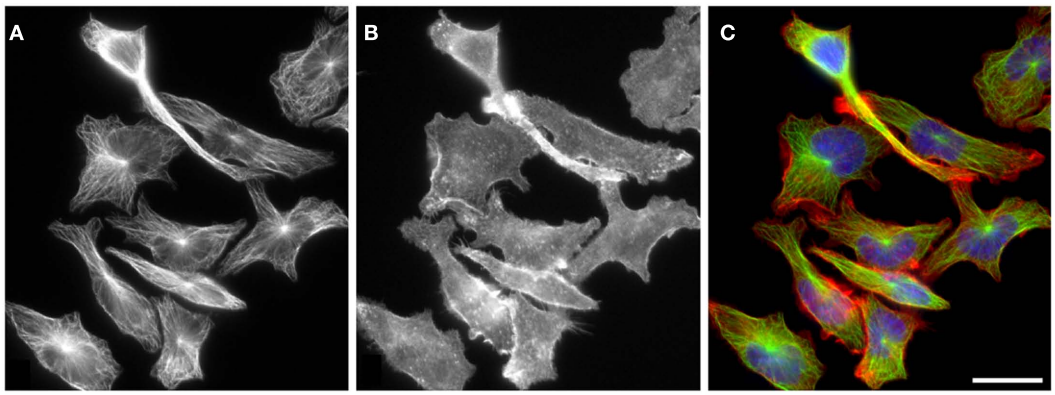

FIGURE 2 | Distribution of microtubules and F-actin in RBL-2H3 cells. Microtubules **(A)** and F-actin **(B)** have distinct subcellular organization in resting interphase RBL-2H3 cells. While microtubule arrays radiate from perinuclear centrosomes, F-actin does not have a single organizing center within the cell and is more concentrated at cell periphery. Cells were fixed with formaldehyde and extracted with Triton X-100 before staining with rabbit antibody to α-tubulin [**(A)**; green] and rhodamine-conjugated phalloidin [**(B)**; red]. DNA was stained with DAPI (blue). Superposition of α-tubulin and F-actin is shown in **(C)**. Scale bar, 20 μm. Photography E. Dráberová (Institute of Molecular Genetics AS CR, Prague).

by motor proteins. They are organized into radial cytoplasmic networks in interphase cells, bipolar spindles in mitotic cells, and midbodies during cytokinesis. The basic building blocks of microtubules are heterodimers of globular α- and β-tubulin subunits, which are arranged in a head-to-tail fashion to form 13 protofilaments that constitute cylindrical and left-handed helical microtubule wall with outer diameter around 25 nm (Kreis and Vale, 1999). Microtubules are thus inherently polar, and contain two structurally distinct ends: a slow-growing minus end, exposing α-tubulin subunits, and a fast-growing plus end, exposing β-tubulin subunits (Nogales and Wang, 2006). Typically, microtubule minus ends are stably anchored in microtubule-organizing centers (MTOC) as centrosomes, whereas the plus ends are highly dynamic and switch between phases of growth and shrinkage. The properties of microtubule can be regulated by incorporation of alternative tubulin isotypes, post-translational modification (PTM) of tubulin subunits, and binding of microtubule associated proteins (MAPs) (Amos and Schlieper, 2005; Verhey and Gaertig, 2007).

Both tubulin subunits are encoded by multiple genes, which are in a large part phylogenetically conserved; eight α-tubulin and seven β-tubulin isotypes were identified in human (Luduena and Banerjee, 2008; Katsetos and Dráber, 2012). The secondary and tertiary structures of the α- and β-monomers are essentially identical, and reflect more than 40% identity over their entire sequence (Nogales et al., 1998). Each tubulin monomer binds one molecule of GTP, non-exchangeably in α-subunit and exchangeably in β-subunit. Most of the studied microtubules appeared to have a seam along their length. This is due to 12-nm helical pitch in combination with the 8-nm longitudinal repeat between αβ-tubulin dimers (Wade and Hyman, 1997).

MICROTUBULE DYNAMICS AND NUCLEATION

Polymerization and depolymerization of microtubules is driven by the binding, hydrolysis, and exchange of GTP on the β-tubulin monomer. GTP hydrolysis is necessary for switching between alternating phases of growth and shrinkage separated by catastrophe (transition from growth to shrinkage) and rescue (transition from shortening to growth) events. Polymerization is initiated from a pool of GTP-loaded tubulin subunits. Growing microtubule sheets maintain a "cap" of tubulin-GTP subunits to stabilize the straight tubulin conformation within the microtubule lattice (Carlier, 1991). A loss of this cap results in rapid depolymerization. The polymerization-depolymerization cycle is completed by exchanging GDP of the disassembly products with GTP (**Figure 3**) (Akhmanova and Steinmetz, 2008). These characteristics result in dynamic instability (Mitchison and Kirschner, 1984), an essential feature of microtubules that allows them to search through the cell for targets, such as the chromosomal kinetochores, the cell cortex, and actin cytoskeleton (Desai and Mitchison, 1997). The stability and dynamics of microtubules are actively regulated by a number of cellular factors as well as a variety of ligands, among them are well-known drugs (e.g., vinblastine, nocodazole and paclitaxel) that potently suppress the dynamic instability of microtubules (Jordan and Kamath, 2007).

One of the key components required for microtubule nucleation and stabilization is the γ-tubulin (Oakley and Oakley, 1989), a highly conserved, but minor, member of the tubulin family concentrated in interphase cells in MTOCs (Stearns et al., 1991). In mitotic cells γ-tubulin appears on spindle poles and along spindle fibers, during cytokinesis it is found in midbodies (Julian et al., 1993; Nováková et al., 1996). γ-Tubulin has also been found to bind to membranous components of the cell (Chabin-Brion et al., 2001; Dryková et al., 2003). Contrary to αβ-tubulin dimers only two functional genes exist in mammalian cells that code very similar γ-tubulins (Wise et al., 2000; Vinopal et al., 2012). The γ-tubulin is associated in complexes with other proteins. The human γ-tubulin small complex (γTuSC) comprises two molecules of γ-tubulin and one molecule each of GCP (γ-tubulin complex protein) 2 and 3. The large γ-tubulin-ring complex (γTuRC) derives from γ-TuSCs by condensation and association with proteins GCP4, GCP5 and GCP6 (Murphy et al., 2001). Soluble γ-tubulin can associate with

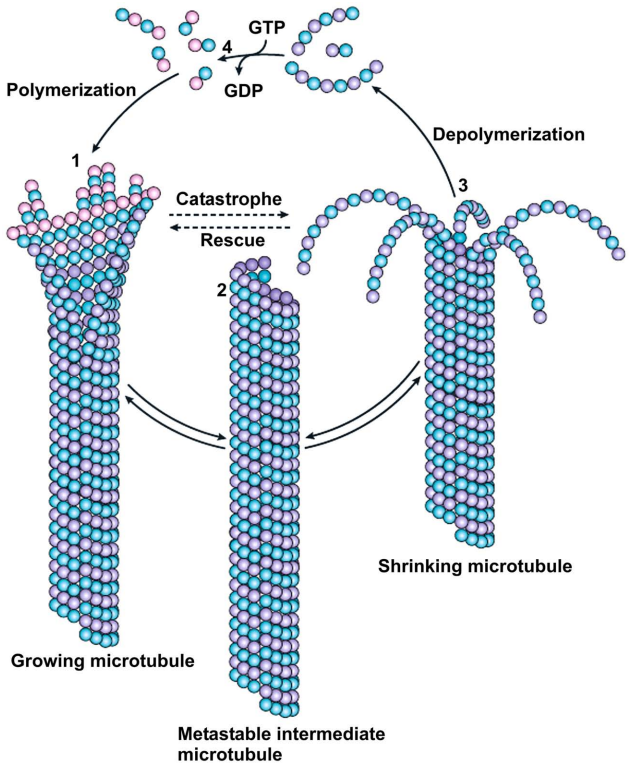

FIGURE 3 | Dynamic instability of microtubules. Polymerization of microtubules is initiated from a pool of GTP-loaded tubulin subunits (pink beads). GTP hydrolysis changes the conformation of the protofilament from a slightly curved tubulin-GTP to a more sharply curved tubulin-GDP structure. The curved tubulin-GDP (lilac beads) is forced to remain straight when it is part of the microtubule wall. Growing microtubule sheets presumably maintain the "cap" of tubulin-GTP subunits to stabilize the straight tubulin conformation within the microtubule lattice (1). Closure of the terminal sheet structure generates a metastable, blunt-ended microtubule intermediate (2) which may pause, undergo further growth or switch to the depolymerization phase. A shrinking microtubule is characterized by fountain-like arrays of ring and spiral protofilament structures (3). The polymerization–depolymerization cycle is completed by exchanging GDP of the disassembly products with GTP (4). Transition from growth to shrinkage is termed catastrophe; transition from shortening to growth is called rescue. Reprinted by permission from Macmillan Publishers Ltd: (Nature Reviews in Molecular Biology; Akhmanova and Steinmetz, 2008).

αβ-tubulin dimers irrespective of the size of γ-tubulin complexes (Sulimenko et al., 2002). Several reports indicated that kinases might be involved in the regulation of γ-tubulin interactions (Kapeller et al., 1995; Vogel et al., 2001; Kukharskyy et al., 2004).

MODULATION OF MICROTUBULE FUNCTIONS BY POSTTRANSLATIONAL MODIFICATIONS AND ASSOCIATED PROTEINS

αβ-Tubulin dimers can be separated according to their isoelectric points to more than 20 isoforms, far more than expected from the number of isotypes that are actually expressed (Wolff et al., 1982; Linhartová et al., 1992). This fact reflects extensive PTMs of both tubulin subunits. Most PTMs of tubulin subunits take place after polymerization into microtubules, and modified tubulins are non-uniformly distributed along microtubules. More than fifteen tubulin PTMs have been described; well characterized PTMs include acetylation, detyrosination, polyglutamylation, and polyglycylation. Diverse PTMs form the biochemical "tubulin code" that can be read by factors interacting with microtubules. Specific microtubule regions can be distinguished biochemically and functionally by the presence of PTMs on tubulins (Westermann and Weber, 2003; Verhey and Gaertig, 2007). It appears that modifications could participate in targeting the molecular motors and MAPs to defined subsets of microtubules inside the cell (Janke and Bulinski, 2011).

Proteins interacting with microtubules are involved in microtubule growth, stabilization, destabilization, and connection of microtubules to other cellular organelles. The mechanochemical ATPases kinesins and dyneins (microtubule motor proteins) use microtubules as pathways for intracellular transport. A wide variety of microtubule regulatory proteins promote the functional diversity of microtubules (Lyle et al., 2009a,b; Wade, 2009). Regulation can occur on many levels, including regulation of tubulin monomer folding or microtubule nucleation. Microtubule stability and dynamics is regulated by a large number of proteins that belongs to microtubule-stabilizing MAPs, microtubule severing proteins, microtubule-disassembly, or assembly promoters and microtubule plus-end tracking proteins (+TIPs) that specifically accumulate at growing microtubule plus ends. To essential +TIPs belongs the end-binding protein 1 (EB1) that directly interacts with tubulin dimers (Dráber and Dráberová, 2012). Subcellular localization of γ-tubulin and EB1 in resting RBL-2H3cells is shown in **Figure 4**.

MICROTUBULES IN ACTIVATED MAST CELLS

CHANGES IN MICROTUBULE ORGANIZATION AND DYNAMICS IN ACTIVATED CELLS

It is well established that agents inhibiting tubulin polymerization suppress the exocytosis (Urata and Siraganian, 1985; Tasaka et al., 1991) and that the movement of secretory granules in mast cells is dependent on intact microtubules (Martin-Verdeaux et al., 2003; Smith et al., 2003; Nishida et al., 2005).

Activation of BMMCs cells leads to changes in topography of microtubules. Compared to resting cells, FcεRI aggregation in BMMCs attached to poly-L-lysine resulted in accumulation of microtubules in cell periphery detectable by immunofluorescence microscopy. Similarly, activation of BMMCs by pervanadate, that mimics the stimulatory effect of antigen, induced enhanced formation of microtubules (Sulimenko et al., 2006). The same effect was also observed when BMMCs were activated and fixed in cell suspension before attachment to glass slides by cytospin (Nishida et al., 2005). When prior to activation the cells were attached to fibronectin, what more closely resembles the natural conditions existing in connective tissue where mast cells are congregated (Galli et al., 2008), prominent protrusions containing microtubules appeared in activated cells (Hájková et al., 2011). Attachment of BMMCs to fibronectin alone failed to generate such protrusions (**Figure 5**).

Microtubule dynamics can be examined by live cell imaging of tagged-EB1. When microtubule plus-end dynamics in cells expressing EB1-GFP was monitored by means of time-lapse

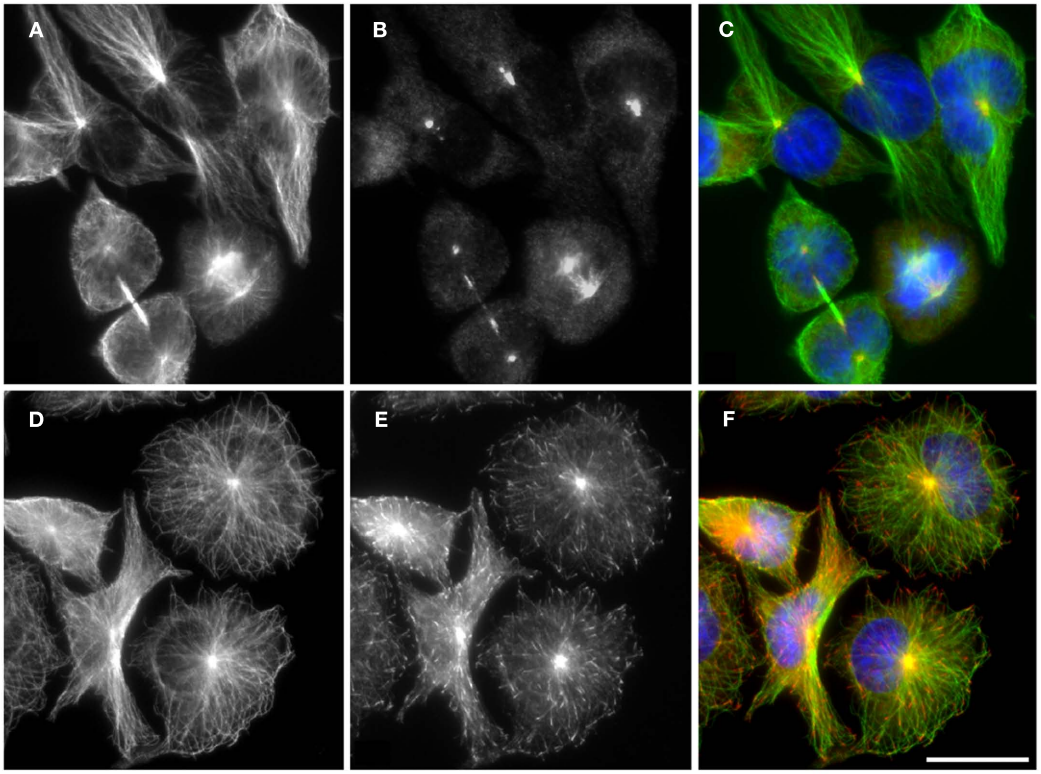

FIGURE 4 | Distribution of microtubule proteins in RBL-2H3 cells. In resting RBL-2H3 cells, microtubules, formed by αβ-tubulin dimers **(A,D)** originate from centrosomes where the γ-tubulin is concentrated **(B)**. Growing microtubules are marked by plus-end tracking protein EB1 **(E)**. Cells were fixed with cold methanol before staining with rabbit antibody to α-tubulin dimer [**(A,D)**; green fluorescence], mouse monoclonal antibody to γ-tubulin [**(B)**, red fluorescence], or monoclonal antibody to EB1 [**(E)**, red fluorescence]. DNA was stained with DAPI (blue). Superposition of α-tubulin and γ-tubulin is shown in **(C)**. Superposition of α-tubulin and EB1 is shown in **(F)**. Scale bar, 20 μm. Photography E. Dráberová (Institute of Molecular Genetics AS CR, Prague).

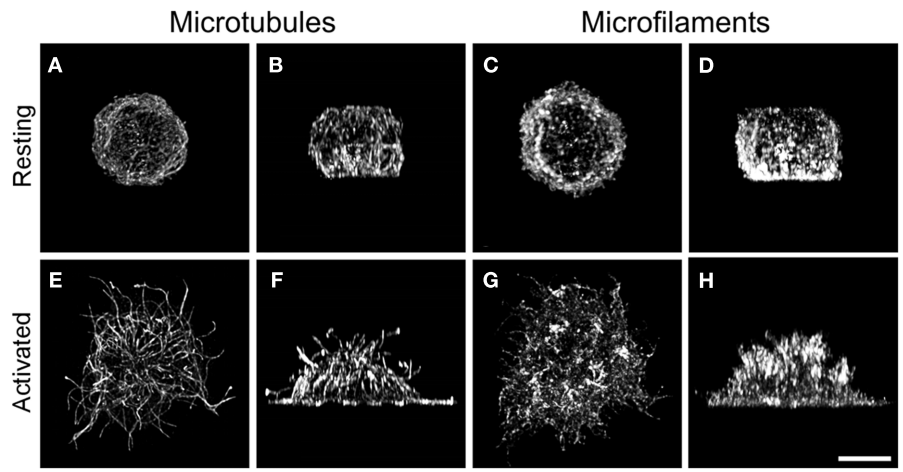

FIGURE 5 | Reorganization of microtubules and F-actin in activated BMMCs. Resting BMMC **(A–D)** or thapsigargin-activated BMMC **(E–H)** attached to fibronectin were fixed in formaldehyde and extracted in Triton X-100. Cells were double label stained for α-tubulin **(A,B,E,F)** and F-actin **(C,D,G,H)**, and examined with laser scanning confocal microscope. The stacks of confocal sections were deconvoluted and subjected to three-dimensional reconstruction using Huygens deconvolution software. The resulting 3-D images are viewed from top of the cells **(A,C,E,G)** and from the plane perpendicular to the plane of cell adhesion **(B,D,F,H)**. The same cell is represented in **(A–D)** or **(E–H)**. Scale bar, 5 μm. Photography Z. Hájková (Institute of Molecular Genetics AS CR, Prague).

imaging using total internal reflection fluorescence microscopy (TIRFM), the number of growing microtubules in the periphery of activated cells was found substantially increased compared to non-activated cells (**Figure 6**). It is known that the engagement of integrins by their ligands activates some signaling pathways that modulate signals originating from other receptors (Schwartz et al., 1995). When mast cells were activated via FcεRI and integrins simultaneously, phosphorylation events were prolonged, and intensified (Lam et al., 2003). Thus, generation of protrusions in activated cells could reflect a response to such integrated signals.

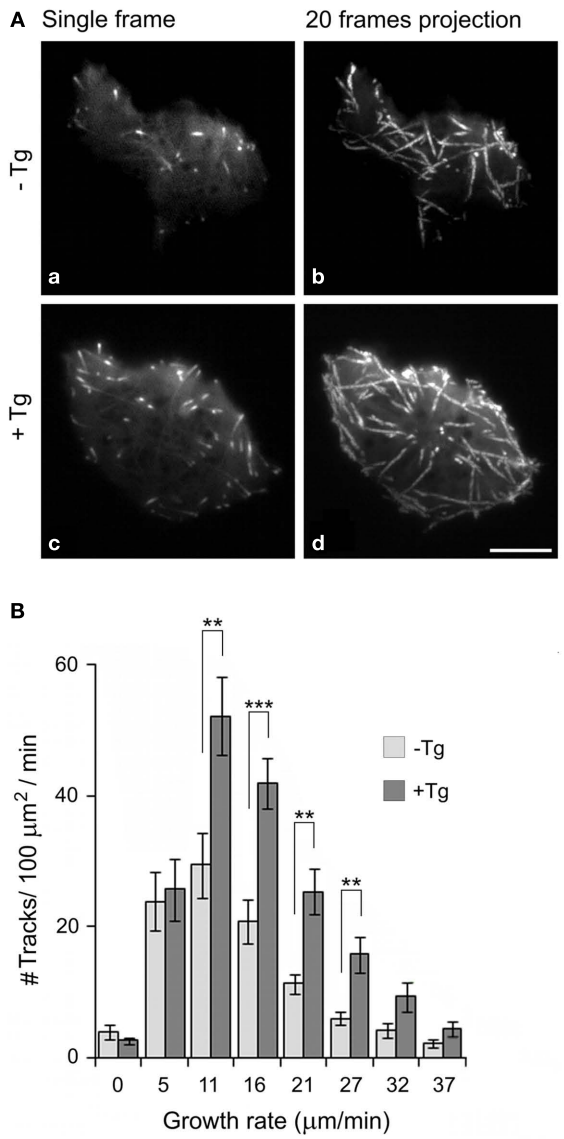

FIGURE 6 | Changes in the number of growing microtubules in cell periphery during mast cell activation as determined by TIRFM time-lapse imaging in living cells. (A) Time-lapse imaging of resting **(a,b)** and thapsigargin-activated **(c,d)** BMMCs expressing EB1-GFP. Still images of EB1 **(a,c)** and tracks of EB1 comets over 20 s created by maximum intensity projection of 20 consecutive frames **(b,d)**. Scale bar, 5 μm. **(B)** Histogram of microtubule growth rates in the cell periphery of resting (–Tg) and thapsigargin-activated (+Tg) cells. A total of 15 different cells were tracked in five independent experiments. Values indicate mean ±SE, $n = 15$ (**$p < 0.01$; ***$p < 0.001$). Reproduced by permission from reference Hájková et al. (2011). Copyright 2011; The American Association of Immunologists, Inc.

STORE-OPERATED Ca^{2+} ENTRY AND MICROTUBULES IN ACTIVATED CELLS

The store-operated Ca^{2+} entry (SOCE) is important for the replenishment of intracellular stores by means of sarco/endoplasmatic reticulum Ca^{2+}-ATPase (SERCA) pumps located in ER membrane (Parekh and Putney Jr, 2005; Smyth et al., 2006). A key component in SOCE is the stromal interacting protein 1 (STIM1; Roos et al., 2005), that represents the Ca^{2+} sensor responsible for communicating the depleted state of intracellular Ca^{2+} compartments to store-operated Ca^{2+} channels. In quiescent cells with ER filled with Ca^{2+}, STIM1 is distributed homogenously throughout ER (Dziadek and Johnstone, 2007) but relocates upon release of Ca^{2+} from ER stores to distinct puncta on ER in close proximity to the plasma membrane (Liou et al., 2005). Aggregated STIM1 activates members of the Orai family of store-operated Ca^{2+} channels, resulting in opening of plasma membrane Ca^{2+} release-activated Ca^{2+} (CRAC) channels and Ca^{2+} influx into the cell (Prakriya et al., 2006). In this way STIM1 serves as a major regulator of SOCE. In addition, transient receptor potential channels (TRPC) and ion exchangers also contribute to the generation of Ca^{2+} signals that may be global or have dynamic (e.g., waves and oscillations) and spatial resolution for specific functional readouts (Ma and Beaven, 2009). SOCE has been described in various cell types including mast cells activated by antigen stimulation, which induces IP_3 mediated Ca^{2+} release from ER stores. Aggregation of FcεRI receptors triggered in RBL cells significant oligomerization of STIM1 and formation of STIM1 puncta near the plasma membrane (Liou et al., 2007). Further study showed that clustering of STIM1 with Orai 1 at plasma membrane of antigen-activated RBL-2H3 cells was depended on the extent of Ca^{2+} store depletion (Calloway et al., 2009). The results pointed to the capability of TRPC proteins to interact with STIM1 and Orai proteins in mast cells. This could add further flexibility to the Ca^{2+} "tool kit" that is available to activated mast cells (Ma and Beaven, 2011).

Colocalization of ER-embedded STIM1 with microtubules has been described for several cell types including DT40 B cells (Baba et al., 2006), HEK293 cells (Mercer et al., 2006; Smyth et al., 2007), and mast RBL-2H3 cells (Calloway et al., 2009). Moreover, comet-like movement of STIM1, resembling +TIPs, was reported in living cells (Grigoriev et al., 2008), and STIM1 was found to contain a short sequence (SxIP) responsible for direct binding to EB1 (Honnappa et al., 2009). Comet-like movement of STIM1 was substantially reduced after addition of thapsigargin (Hájková et al., 2011), a compound that inhibits SERCA (Thastrup et al., 1990). This is in agreement with the impaired association of STIM1 with microtubules observed in antigen-activated RBL-2H3 cells (Calloway et al., 2009). Data on fluorescence resonance energy transfer (FRET) imaging of EB1 and STIM1 showed that STIM1 dissociated from EB1 and associated with SERCA upon store depletion

of Ca^{2+}. The process seems to be reversible, since the replenishment of intracellular Ca^{2+} stores also restored the STIM1-EB1 interactions (Sampieri et al., 2009). Moreover, no effect on SOCE was observed in cells with depleted EB1 (Grigoriev et al., 2008). Considering these findings together, it is likely that interaction of STIM1 with EB1 on growing microtubules is not essential for the transport of STIM1 to plasma membrane during mast cell activation.

After depletion of intracellular Ca^{2+} stores, STIM1 accumulates into puncta, i.e., discrete subregions of ER located in a close proximity (10–25 nm) to plasma membrane (Cahalan, 2009). STIM1 puncta are formed several seconds before the opening of Ca^{2+} channels (Wu et al., 2006), and it could therefore be inferred that microtubules are involved in this process. However, microtubule disruption by nocodazole had no effect on puncta formation in activated BMMCs, while the comet-like movement of STIM1 was abolished (Hájková et al., 2011). This is in line with observations that uptake of extracellular Ca^{2+} was only partially inhibited in nocodazole-pretreated and thapsigargin-activated BMMCs. This may suggest that STIM1 aggregation beneath the plasma membrane and subsequent opening of Ca^{2+} release-activated Ca^{2+} channels do not require intact microtubules in activated mast cells (Hájková et al., 2011). Previous studies often reported discordant effects of nocodazole treatment on SOCE or I_{CRAC}, the current most frequently associated with SOCE, in various cell types. While no effect of nocodazole was observed in RBL-1 (Bakowski et al., 2001; Smyth et al., 2007) and DT40 cells (Baba et al., 2006), significant inhibition was demonstrated in other cell types, such as RBL-2H3, BMMCs (Oka et al., 2005), and HEK 293 (Smyth et al., 2007). It appears that other factors next to cell type, like the treatment protocol or the method of Ca^{2+} depletion, might modify the results of the experiments. It is also possible that microtubules play a supporting role in SOCE signaling by optimizing the location of ER containing STIM1 before cell activation (Smyth et al., 2007; Galan et al., 2011).

Protrusions containing microtubules were found in BMMCs stimulated by three types of activators that induced depletion of Ca^{2+} from internal stores (FcεRI aggregation, pervanadate, or thapsigargin treatment). Formation of protrusions was inhibited in cells with decreased level of STIM1 and with correspondingly reduced influx of extracellular Ca^{2+}. Protrusions in BMMCs with depleted level of STIM1 were restored after introduction of human STIM1. These data together with the absence of protrusions in cells activated in Ca^{2+}-free media suggest that STIM1 regulated Ca^{2+} influx plays a crucial role in generation of these microtubule enriched structures. Changes in the concentration of cytoplasmic Ca^{2+}also affected a microtubule plus-end dynamics and altered chemotactic response. Altogether these results support the concept of a tight crosstalk between microtubular network and Ca^{2+} signaling machinery in the course of mast cell activation (Hájková et al., 2011). The presence of aggregated STIM1 in protrusions could help organize Ca^{2+} release-activated Ca^{2+} channels (Cahalan, 2009) and open locally these channels to bring about SOCE. These interactions may be subjected to modulations by Ca^{2+} channel regulators, such as calmodulin (Mullins et al., 2009) or CRACR2A, a cytosolic Ca^{2+} sensor that stabilizes CRAC channels (Srikanth et al., 2010).

γ-TUBULIN IN ACTIVATED MAST CELLS

The formation of microtubules can be effectively regulated by microtubule nucleation, a process where γ-tubulin plays a key role. It was reported that Lyn kinase, a major Src-family kinase in RBL-2H3 cells (Eiseman and Bolen, 1992), forms complexes containing γ-tubulin and phosphotyrosine proteins in these cells activated by FcεRI aggregation or by an exposure to pervanadate (Dráberová et al., 1999). However, Lyn kinase is dispensable for the formation of functional γ-tubulin complexes, as indicated by normal topography of microtubules in $Lyn^{-/-}$ BMMCs. Tyrosine-phosphorylated proteins were found to be associated with immunoprecipitated γ-tubulin in resting $Lyn^{-/-}$ BMMCs, and the level of tyrosine phosphorylation of these proteins substantially increased after activation. Immunoprecipitation experiments and *in vitro* kinase assays combined with specific inhibitors revealed that Fyn and Syk kinases in complexes containing γ-tubulin are capable of phosphorylating various substrates (Sulimenko et al., 2006). Interaction of γ-tubulin complexes with Fyn was confirmed by pull-down experiments, where γ-tubulin complex bound to SH2 domain of Fyn kinase in a phosphotyrosine dependent manner. Similar binding to SH2 domains of Fyn or Src kinases has also been described in mouse embryonal carcinoma cells (Kukharskyy et al., 2004). Although γ-tubulin possesses a potential binding site for SH2 domain, experiments with several candidate synthetic peptide inhibitors failed to confirm a direct binding of γ-tubulin to this domain. Thus, the association with SH2 domain is probably mediated via adaptor-like tyrosine-phosphorylated protein(s) (Sulimenko et al., 2006). The Fyn kinase, like other Src-family kinases, is commonly involved in the formation of multi-protein complexes engaged in interaction with the SH2 and SH3 domains. It is therefore likely that association of Src-family kinases with γ-tubulin is mediated through other proteins that might be important for microtubule nucleation. Significance of Src kinase for microtubule nucleation from centrosomes was ascertained by microtubule regrowth experiments on human fibroblasts adherent to fibronectin (Colello et al., 2010). Src-family kinases could regulate the recruitment of γ-tubulin to the centrosome by different mechanisms. They can either phosphorylate γTuRC proteins to promote the assembly of the complex, or may regulate the association or activity of NEDD1/GCP-WD, a protein that is required for the centrosomal recruitment of γTuRC (Lüders et al., 2006). Alternatively, kinases may indirectly affect this process by regulating the assembly of the pericentriolar matrix (PCM) surrounding centrioles. Identification of Src-family kinase substrates that promote the recruitment of γ-tubulin to the centrosome and microtubule nucleation will be an important step forward in the elucidation of the mechanisms involved.

The molecular mechanism of the association of Syk kinase with γ-tubulin is not fully understood. In RBL-2H3 cells as well as in BMMCs, Syk is one of the preferable substrates for Lyn kinase (Jouvin et al., 1994). However, even in $Lyn^{-/-}$ BMMCs there is still some phosphorylation of Syk on tyrosine, which is dependent on FcεRI activation (Parravicini et al., 2002). Because the Src-family selective tyrosine kinase inhibitor PP2 inhibited phosphorylation of Syk in pervanadate-activated $Lyn^{-/-}$ BMMCs, and the Syk-selective tyrosine kinase inhibitor piceatannol reduced

phosphorylation of proteins in γ-tubulin immunocomplexes, it is likely that it is the crosstalk between Fyn and Syk which is responsible for tyrosine phosphorylation of proteins associated with γ-tubulin immunocomplexes in $Lyn^{-/-}$ BMMCs (Sulimenko et al., 2006).

There are reports pointing to the localization of Fyn and Syk kinases in centrosomal region. Fyn kinase was found in centrosomes in myelocytic leukemia cell line HL-60 (Katagiri et al., 1993) and in human T lymphocytes (Ley et al., 1994). Moreover, in human Jurkat T lymphocytes tubulin phosphorylated on tyrosine interacted with SH2 domain of Fyn kinase (Marie-Cardine et al., 1995). Syk was located at the centrosomes in B lymphocytes (Navara et al., 1999). Thus, tyrosine phosphorylation of centrosomal proteins by Fyn and Syk kinases might be the process linking microtubules to early activation events in mast cells.

Tubulin has been shown to serve as a substrate for Syk kinase *in vivo* (Peters et al., 1996). Syk can phosphorylate both soluble tubulin (Fernandez et al., 1999) and tubulin in microtubules (Faruki et al., 2000). Syk phosphorylates α-tubulin on the conserved tyrosine residue (Tyr432) and Syk-selective inhibitor piceatannol blocks the receptor-stimulated tubulin phosphorylation in B lymphocytes (Peters et al., 1996) as well in BMMCs (Sulimenko et al., 2006). However, phosphorylation of tubulin by Syk did not have any profound effect on microtubule assembly in pervanadate-treated cells (Faruki et al., 2000). Besides, phosphorylation of tubulin by Src kinase did not cause any significant changes in microtubule polymer (Simon et al., 1998). It is therefore unlikely that phosphorylation of tubulin dimers plays a key role in the increase of microtubule formation in activated mast cells (Sulimenko et al., 2006).

It has been repeatedly reported that γ-tubulin is phosphorylated (Vogel et al., 2001; Stumpff et al., 2004; Alvarado-Kristensson et al., 2009). Phosphorylation of the γ-tubulin residue Tyr 445, which is invariably present in all γ-tubulins, was described and a mutation of this residue changed the microtubule dynamics (Vogel et al., 2001). Similarly phosphorylation of multiple serines on γ-tubulin can regulate microtubule organization (Lin et al., 2011). There are other data that point to an association of γ-tubulin with kinases. Phosphoinositide-3-kinase (PI3K) binds to γ-tubulin (Kapeller et al., 1995), and the regulatory subunits of PI3K interacts with γ-tubulin in various cell types (Inukai et al., 2000; Macurek et al., 2008), including BMMCs (V. Sulimenko, unpublished data). Collectively these data suggest that kinases take part in the regulation of γ-tubulin function(s). This could lead to changes in nucleation properties of centrosomes or alternatively to an enhancement of non-centrosomal microtubule nucleation.

SIGNALING PATHWAYS FOR STABILIZATION OF THE PLUS ENDS OF MICROTUBULES

The formation of microtubules can also be regulated by stabilizing their plus ends. It has been shown that the Fyn/Gab2/RhoA signaling pathway, though not the Lyn/SLP-76, plays a critical role in microtubule-dependent degranulation of mast cells, and that RhoA kinase could be involved in stabilization of the plus ends of microtubules (Nishida et al., 2005). It is known that an important role in stabilization of growing microtubules is to be assigned to the +TIPs, whose interactions with microtubules are regulated by phosphorylation (Akhmanova and Steinmetz, 2008; Galjart, 2010). Ca^{2+}-dependent kinases [e.g., conventional protein kinases C (cPKC); Ca^{2+}/calmodulin-dependent kinases] or phosphatases (e.g., PP2B) might participate in the regulation of microtubule stability in activated BMMCs. It has been reported that Ca^{2}-dependent activation of Rac (from the Rho family of small GTPases) depends on the activity of cPKC (Price et al., 2003). It was demonstrated that FcεRI stimulation of BMMCs activated RhoA (Nishida et al., 2005). Interestingly, this GTPase participates in stabilization of microtubule plus ends through its target mDia (Palazzo et al., 2001). Microtubule dynamics may also be influenced by +TIPs that interact at cell periphery with cytoskeletal scaffold IQ domain GTPase-activating protein1 (IQGAP1) which sequesters calmodulin and Rac. An increase in Ca^{2+} liberates calmodulin and IQGAP1 interacts with the A-kinase anchoring protein 220 (AKAP220). This leads to association of IQGAP1 with factors that modulate microtubule dynamics, e.g., CLIP-associated protein-2 (CLASP2). Recruitment of CLASP2 to AKAP220/IQGAP1 complex is regulated by glycogen synthase kinase-3β (GSK3β) and cAMP-dependent protein kinase (PKA) (Logue et al., 2011). It has been shown that FcεRI stimulation of BMMCs triggered the formation of microtubules in a manner independent of Ca^{2+} (Nishida et al., 2005). On the other hand, other results demonstrated Ca^{2+}-dependent formation of protrusions containing microtubules (Hájková et al., 2011). This discrepancy could be attributed to differences in cell activation (absence or presence of integrin engagement) and various methods of preparation of samples for microscopic evaluation. However, it is also possible that the initial stages of microtubule formation as well as the transport of granules along microtubules are Ca^{2+}-independent, but that later stages of activation and formation of microtubule protrusions depend on sustained influx of Ca^{2+}.

Ras guanyl nucleotide-releasing proteins (RasGRPs) belong to a family of proteins that are recruited to the plasma membrane by binding to DAG for subsequent activation of Ras family proteins. GTP-bound Ras has been shown to interact with the catalytic p110 subunit of PI3K, and to induce its activation (Kodaki et al., 1994). PI3K then stimulates Rho GTPases (Kuehn et al., 2010). It was demonstrated that FcεRI activated RasGRP1-depleted BMMCs were defective in RhoA activation, microtubule formation and granule translocation. RasGRP1 could thus play an important role in the regulation of microtubule formation in activated mast cells (Liu et al., 2007).

MICROFILAMENTS

MICROFILAMENTS AND ACTINS

Microfilaments (actin filaments) are essential for a number of cell functions, such as maintenance of cell shape, cell division, migration, junction formation, and intracellular vesicle trafficking powered by motor proteins. Microfilaments are formed by two-stranded helical polymers with a diameter of ∼7 nm. Actin filaments are assembled into two types of structures – bundles and networks. The basic component of microfilaments is a globular protein actin (called G-actin), which forms filaments (called F-actin). The molecule of actin is folded into two domains stabilized

by an adenine nucleotide lying in between. The uniform orientation of asymmetrical subunits along the polymer causes polarization of actin filament with fast-growing plus-end (or barbed end) and slow-growing minus end (or pointed-end). Microfilaments are dynamic structures; subunits can be added or lost on both ends of the polymer. The different rate constants for association and dissociation depend on nucleotide bound to the monomer. Autocatalyzed hydrolysis of ATP to ADP in F-actin reduces the binding strength and modifies the behavior of microfilaments, including their affinity to regulatory proteins (Kreis and Vale, 1999).

Actin is one of the most highly conserved proteins in evolution. It is encoded by multiple genes. Mammals have six genes, and each encodes one protein isoform. Four of them, are expressed primarily in muscles. The remaining two isoforms (β_{cyto}-actin, γ_{cyto}-actin) are expressed ubiquitously. All isoforms possess very similar amino acid sequences, with no isoform sharing less than 93% identity with any other isoform (Perrin and Ervasti, 2010). Although actin isoforms can copolymerize within the cells they are usually sorted out in different structures and perform different cellular functions due to interactions with specific subsets of actin-binding proteins (dos Remedios et al., 2003).

In contrast to microtubules, microfilaments do not have one prominent nucleation center. They can form tight bundles making up the core of microvilli or can be arranged in a less-ordered network as in the cell cortex. In dividing cells they form the contractile ring that is important for cytokinesis. In migrating cells, a branched network of actin filaments is found in the lamellipodia, broad and flat protrusions at the leading edge of moving cells. The thin cellular processes extending from lamellipodium and containing parallel bundles of actin filaments are called filopodia. Many cells have contractile filaments called stress fibers that consist of microfilaments, myosin II motor proteins and other interacting proteins. The ends of stress fibers anchor to the cell membrane in focal adhesions, cell-to-substrate adhesion structures accountable for a strong attachment to substrate (Parsons et al., 2010). There are three categories of stress fibers: ventral stress fibers that are attached to focal adhesions at both ends, dorsal stress fibers that are attached to focal adhesions typically at one end, and transverse arcs that do not directly attach to focal adhesions (Naumanen et al., 2008). Microfilaments are also important for the formation of transient membrane surface structures as podosomes and invadopodia, which attach cells to the extracellular matrix, and take part in the generation of circular dorsal ruffles (Buccione et al., 2004). The stability and dynamics of actin structures is regulated by a variety of ligands including drugs and toxins. Phalloidins bind along the sides of actin filaments, preventing their depolymerization. Phalloidin labeled with a fluorophore is often used for visualization of microfilaments. Jasplakinolides are other actin-filament stabilizers. On the other hand, cytocholasins cap the barbed end of microfilaments, and latrunculins sequester actin monomers. These processes lead to inhibition of microfilament formation (Kustermans et al., 2008).

ACTIN-BINDING PROTEINS REGULATE MICROFILAMENT ORGANIZATION AND DYNAMICS

Actin binds a substantial number of associated proteins. They can be divided into several functional groups. The first group contains monomer-binding proteins that sequester G-actin and prevent its polymerization (e.g., profilin). The second group covers filament-depolymerizing proteins (e.g., ADF/cofilin). The third group includes filament end-binding proteins that cap the ends of actin filament and prevent the exchange of monomers at the pointed-end (e.g., tropomodulin) and at the barbed end (e.g., CapZ). The fourth group contains filament severing proteins (e.g., gelsolin). The fifth group consists of cross-linking proteins that contain at least two binding sites for F-actin, facilitating thus the formation of filament bundles, branching filaments, and three-dimensional networks (e.g., α-actinin, vilin, fimbrin, Arp2/3). In the sixth group are stabilizing proteins that bind to the sides of actin filaments and prevent depolymerization (e.g., tropomyosin). Finally, the seventh group consists of motor proteins that use F-actin as a track upon which to move (e.g., myosins) (dos Remedios et al., 2003). Actin-binding proteins are functionally not limited to one class; for example, gelsolin is capable of severing and capping the barbed end of actin filaments.

Cortical F-actin is connected to the plasma membrane through an array of closely related cytoplasmic proteins of the ERM (Ezrin, Radixin, Moesin) family. In addition to their role in binding filamentous actin, ERMs regulate the signaling pathways through their ability to bind transmembrane receptors and link them to downstream signaling components. ERMs thus serve as scaffolds to facilitate efficient signal transduction on the cytoplasmic face of the plasma membrane (Neisch and Fehon, 2011).

Inside the cells, the *de novo* nucleation of actin filaments from monomers require the participation of actin-nucleating proteins. These proteins fall into three main families: the Arp2/3 complex and its nucleation promoting factors (NPFs), formins, and tandem-monomer-binding nucleators. They have important roles in many essential cellular processes. The Arp2/3 complex is composed of evolutionarily conserved subunits including the actin-related proteins Arp2, Arp3, and five additional subunits ARPC1–5. The Arp2/3 complex by itself is an inefficient nucleator, and its activation requires binding to the sides of actin filaments and to NPF (Goley and Welch, 2006). Among the well characterized NPFs are Wiskott-Aldrich syndrome protein (WASP), Wiskott-Aldrich syndrome protein-family verprolin homologous protein (WAVE), WASP and Scar homolog (WASH), and the more recently identified WASP homolog associated with actin, membranes, and microtubules (WHAMM; Firat-Karalar and Welch, 2011). Once activated, the Arp2/3 complex nucleates the formation of new filaments that extend from the sides of existing filaments at a 70° to form a Y-branched network. In contrast, nucleation by formins (e.g., mammalian Diaphanous formin, mDia) and tandem-monomer-binding nucleators (e.g., adenomatous polyposis coli, APC) leads to unbranched filaments (Firat-Karalar and Welch, 2011). Arp2/3 complex activity is inhibited by coronin 1b that promotes debranching of actin filaments and recycling of the Arp 2/3 complex (Cai et al., 2007). Similarly, cofilin also dissociates Arp2/3 complex and branches from actin filaments (Chan et al., 2009).

Rho GTPases AS KEY REGULATORS OF MICROFILAMENTS

Diverse cell-surface receptors trigger global structural rearrangements of actin cytoskeleton in response to external signals. These

signals converge inside the cell on a group of closely related monomeric GTPases that belong to the Rho protein family. The three prevailing subclasses are Rho, Rac, and Cdc42. Rho activates the formation of stress fibers and focal adhesions (Ridley and Hall, 1992), Rac activates the formation of lamellipodia and membrane ruffles (Ridley et al., 1992) and Cdc42 activates the formation of filopodia (Nobes and Hall, 1995). In each case the active form of protein binds GTP. Down regulation of Rho GTPases involves GTPase-activating proteins (GAPs) that facilitate GTP hydrolysis. Activation of Rho proteins is promoted by guanine nucleotide exchange factors (GEFs) that control the release of GDP from the Rho protein and its replacement with GTP. GEFs are often activated through signal cascades initiated via plasma membrane receptors. Guanine nucleotide dissociation inhibitors (GDI) bind to Rho and prevent GDP/GTP exchange (Bement et al., 2006). GTPases also function as molecular switches in multiple signaling processes including regulation of phospholipase D (PLD) and PI3K (Bishop and Hall, 2000).

The downstream targets of Rho GTPases include kinases, formins, families of WASP proteins, and other scaffolding molecules. Of these major subclasses, the Rho-associated coiled-coil kinase (ROCK), the p21-activated kinase (PAK), the mDia, and proteins of the WASP and WAVE families, have direct effects on actin cytoskeleton rearrangements. In addition to activation of formins promoting actin-filament growth, Rho GTPase also promotes myosin actin interactions inducing development and contraction of stress fibers through ROCK. ROCK has been shown to directly phosphorylate a number of actin cytoskeleton regulators including myosin II light chain (MLC), myosin light chain phosphatase (MLCP), and LIM-motif containing kinase (LIMK). Direct phosphorylation of MLC or MLCP has an immediate impact on the level of phosphorylated myosin light chain, which enhances the contractility. Activation of LIMK by ROCK is linked to phosphorylation and inhibition of cofilin, thereby regulating the actin-filament turnover (Spiering and Hodgson, 2011). Feeding directly into this pathway, Rac and Cdc42 activate PAK and consecutively also LIMK. Rac activates the ARP2/3 complex through the WAVE complex, and CDC42 induces actin polymerization by binding to WASP (Heasman and Ridley, 2008). Localization of WASP can be regulated by interaction with other binding partners including Grb2, likely targeting the molecule to sites of receptor stimulation and active actin cytoskeleton remodeling (Carlier et al., 2000). Interestingly, mDia proteins through association with +TIPs has been shown to stabilize microtubules, tying the actin cytoskeleton rearrangements to the microtubule dynamics (Bartolini et al., 2008). Downstream effector targets of the Rho family of GTPases for generation of actin cytoskeletal structures are schematically shown in **Figure 7**.

MICROFILAMENTS IN ACTIVATED MAST CELLS

CHANGES IN ACTIN-FILAMENT ORGANIZATION

Dramatic reorganization of actin cytoskeleton has been observed in mast cells activated by FcεRI aggregation. The F-actin content of the detergent-extracted cell matrices in RBL-2H3 cells decreased during the first 10–30 s after antigen binding and then increased within 1 min to almost double the control levels. The antigen-stimulated increase in F-actin coincided with the transformation of cell-surface from a finely microvillous to a highly folded topography, and with increased cell spreading (Pfeiffer et al., 1985). Enhanced formation of F-actin after cell triggering was reported in several other studies using RBL-2H3 cells (Apgar, 1994; Frigeri and Apgar, 1999; Holowka et al., 2000; Tolarová et al., 2004), RPMCs (Pendleton and Koffer, 2001), and BMMCs (Tumová et al., 2010).

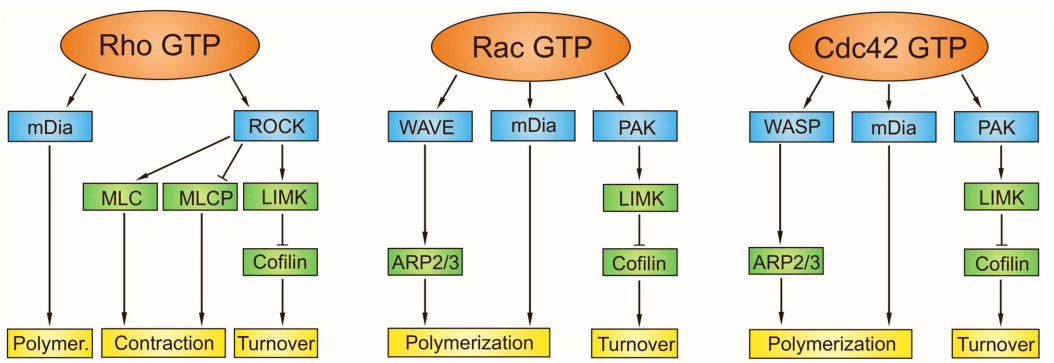

FIGURE 7 | Downstream effector targets of Rho family GTPases for generation of actin cytoskeletal structures. The downstream targets of active Rho GTPases, with bound GTP, include kinases (p21-activated kinase, PAK; Rho-associated coiled-coil kinase; ROCK) and nucleation promoting factors (mammalian Diaphanous formin, mDia; Wiskott-Aldrich syndrome protein, WASP; Wiskott-Aldrich syndrome protein-family verprolin homologous protein, WAVE). While nucleation by mDia produces unbranched actin filaments, WASP and WAVE interact with Arp2/3 complex and generate branched microfilaments. PAK phosphorylates LIM-motif containing kinase (LIMK), that in turn phosphorylates and inhibits cofilin thereby regulating the actin-filament turnover. Besides stimulating the actin-filament growth, Rho GTPase promotes myosin actin interactions through ROCK. The ROCK phosphorylates a number of actin cytoskeleton regulators including myosin II light chain (MLC), myosin light chain phosphatase (MLCP), and LIMK. Direct phosphorylation of MLC or MLCP has an immediate impact on the level of phosphorylated myosin light chain, which contributes to contractility. Rho mainly activates the formation of stress fibers and focal adhesions, Rac activates the formation of lamellipodia and membrane ruffles, and Cdc42 activates the formation of filopodia.

Sensitization with IgE alone resulted in an enhancement of cortical F-actin in RBL-2H3 cells (Oka et al., 2004) and BMMCs (Allen et al., 2009).

Inhibitors that disrupt microfilaments, such as latrunculin and cytochalasin D, did not by themselves cause any degranulation in RBL-2H3 cells, but they enhanced the FcεRI-mediated degranulation. However, latrunculin was ineffective if pervanadate was used for activation. This suggests that microfilaments are indispensible for the downstream signaling cascade (Frigeri and Apgar, 1999). Good correlation was found between inhibition of actin polymerization and increased activity of tyrosine kinase and phospholipase. Microfilaments appear to down-regulate the response by affecting the level of receptor tyrosine phosphorylation (Frigeri and Apgar, 1999; Holowka et al., 2000). Similarly, FcεRI-mediated degranulation was enhanced in BMMCs treated with latrunculin B, while jasplakinolide slightly inhibited β-hexosaminidase release (Nishida et al., 2005). It was proposed that actin controls/modulates a multitude of signaling steps leading from the FcεRI aggregation to the final degranulation (Pendleton and Koffer, 2001; Oka et al., 2002; Andrews et al., 2008).

Microfilaments are crucial not only in early stages of mast cell activation but also in later exocytosis steps. Mast cell exocytosis is accompanied with extensive reorganization of the actomyosin cortex, and FcεRI stimulation triggers F-actin ring disassembly in a Ca^{2+}-dependent manner (Nishida et al., 2005). Both positive and negative roles have been suggested for actin in exocytosis. Actomyosin may facilitate the transport and/or extrusion of secretory vesicles, while cortical F-actin may serve as a barrier preventing the access of vesicles to the plasma membrane (Pendleton and Koffer, 2001). Alternatively, cortical F-actin may act as a barrier between the reserve and the release-ready secretory vesicle pools. F-actin disassembly may cause disruption of this barrier (Malacombe et al., 2006).

The combination of atomic force microscopy and laser scanning confocal microscopy enabling simultaneous visualization and correlation of membrane morphology with actin arrangement revealed that reorganization of microfilaments in activated BMMCs depended on the type of stimuli used for cell activation. In cells triggered by FcεRI aggregation, characteristic membrane ridges formed in accordance with the rearrangement of underlying F-actin networks. On the other hand, in BMMCs stimulated just only by poly-L-lysine coated on glass surface, lamellopodia and filopodia were observed in association with the F-actin assemblies at and near the cell periphery, whereas "craters" occurred on the central membrane lacking F-actin (Deng et al., 2009). Actin reorganization in activated cells is also strongly modulated by cell adherence to proteins of extracellular matrix (e.g., fibronectin) followed by integrin signaling (Hamawy et al., 1992, 1994). It was reported that co-stimulation of FcεRI and chemokine receptor 1 resulted in generation of long and thin tubes formed from plasma membrane (cytonemes). Cytonemes containing F-actin may participate in intercellular communication during allergic and inflammatory response (Fifadara et al., 2010). Organization of F-actin in non-activated and thapsigargin-activated BMMCs attached to fibronectin is illustrated in **Figure 5**.

Rho GTPases IN REGULATION OF ACTIN FILAMENTS IN ACTIVATED CELLS

In mast cells, small Rho-related GTPases are essential to the exocytotic function (Price et al., 1995; Brown et al., 1998). Expression of dominant negative mutant forms of the Cdc42 and Rac1 in RBL-2H3 cells substantially affected the microfilament reorganization. Inhibition of Cdc42 function decreased cell adhesion, interfered with FcεRI-induced actin plaque assembly and reduced the recruitment of vinculin at the cell-substratum interface. On the other hand, the inhibitory Rac1 mutant abolished FcεRI-mediated membrane ruffling. The expression of inhibitory forms of either Cdc42 or Rac1 significantly inhibited the antigen-induced degranulation. Cdc42 and Rac1 thus control distinct pathways downstream of FcεRI engagement leading to microfilament organization. Both pathways are decisively engaged during the degranulation response induced by FcεRI aggregation (Guillemot et al., 1997). Mast cells derived from Rac 2-deficient mice showed defects in actin-based functions, including adhesion, migration, and degranulation (Yang et al., 2000).

Rho was found to be responsible for *de novo* actin polymerization in RPMCs (Norman et al., 1994), and it also participated in the control of cortical actin disassembly (Sullivan et al., 1999). Stimulation of BMMCs by FcεRI aggregation resulted in an increased activity of Rac (1,2,3) but decreased activity of RhoA followed by slow recovery of the latter. It was suggested that active RhoA may be necessary to initiate the secretory process (perhaps by activating a fusogen), and its immediate subsequent inactivation may be required for cell attachment (Arthur et al., 2000) and/or F-actin disassembly (Norman et al., 1996). The later increase in RhoA activity would then be required for the formation of newly polymerized actin inhibiting exocytosis and promoting cell spreading and migration (Struckhoff et al., 2010). In the pathway utilizing RhoA and ROCK, the adaptor protein NTAL could serve as a positive regulator of actin polymerization and cell spreading (Tumová et al., 2010).

Proteins that become activated during the c-Kit signaling include PI3K and Rac; activation of this pathway triggers actin reorganization. An important PI3K-dependent GEF interacting with Rac is switch-associated protein 70 (SWAP-70), that affects ruffle formation in activated cells. SWAP-70-deficient BMMCs had impaired Rac function and defects in adhesion, migration, and degranulation. Stimulated mutant cells displayed filopodia-like protrusions possibly indicating a shift in balance between Rac and Cdc42 (Sivalenka and Jessberger, 2004). Enhanced activity of PI3K in c-Kit activated cells leads to activation of Bruton's tyrosine kinase (Btk) and Rac. Btk-deficient BMMCs had defects in Rac activation and F-actin rearrangement resulting in impaired migration and chemotaxis. The Btk is not just an important upstream regulator of Ras GTPase but integrates in addition the signaling pathways from activated c-Kit, GPCR, and aggregated FcεRI (Kuehn et al., 2010).

Wiskott-Aldrich syndrome protein interacting protein (WIP) is another important regulator of the actin cytoskeleton that inhibits Cdc42-mediated activation of WASP. Most of WASP in lymphocytes appear to be sequestered with WIP, and binding to WIP is essential for the stability of WASP (Ramesh and Geha, 2009).

More efficient generation of actin filaments was detected in WIP-deficient BMMCs activated by FcεRI aggregation (Kettner et al., 2004). Impaired actin polymerization, cell spreading, formation of ruffles and degranulation were observed in WASP-deficient BMMCs stimulated by FcεRI signaling (Pivniouk et al., 2003). All these data strongly indicate that Rho GTPases and their effectors have a major role in organization of microfilaments in activated mast cells.

REGULATION OF F-ACTIN IN ACTIVATED CELLS BY CHANGES IN Ca^{2+} CONCENTRATION

Rapid dissolution of cortical F-actin following FcεRI stimulation of mast cells has been interpreted in many studies as a crucial step in facilitating the granule-plasma membrane fusion necessary for the release of granule contents to extracellular environment (Nielsen et al., 1989; Narasimhan et al., 1990; Frigeri and Apgar, 1999; Oka et al., 2004; Deng et al., 2009). Cortical F-actin disassembly is, however, also required for the motile responses. Numerous studies further also revealed that the influx of Ca^{2+} from extracellular space is a prerequisite for cortical F-actin depolymerization (Koffer et al., 1990; Nishida et al., 2005; Shimizu et al., 2009; Suzuki et al., 2010). In contrast, other reports concluded that cortical F-actin disassembly can be induced by both Ca^{2+}-dependent and Ca^{2+}-independent pathways (Sullivan et al., 1999; Guzman et al., 2007). More prominent F-actin depolymerization was observed in NTAL-depleted BMMCs simultaneously activated by FcεRI aggregation and SCF, when compared to activated BMMCs controls. However, the influx of Ca^{2+} was basically unchanged (Tumová et al., 2010).

One of the possible mechanisms of Ca^{2+}-dependent cortical F-actin depolymerization is based on activation of severing protein gelsolin (Borovikov et al., 1995). Another actin-severing protein from the gelsolin family, strongly upregulated in mouse mast cells, is adseverin (D5) (Robbens et al., 1998). Calmodulin also plays an important role in Ca^{2+}-dependent disassembly of microfilaments. This multifunctional protein transduces Ca^{2+} signals by binding the Ca^{2+} and interacting then with various target proteins. Ca^{2+}-calmodulin causes changes in the activity of target proteins. In this connection, it should be mentioned that the disassembly of the actin cortex in RPMC is preceded by acto-myosin-II-based contraction, activated by Ca^{2+}-calmodulin/myosin light chain kinase (MLCK) that phosphorylates the regulatory light chain of myosin II (MLC), activating the motor protein. Inhibition of myosin II significantly inhibited the Ca^{2+}-evoked cortical actin disassembly. Thus, calmodulin plays an essential role in Ca^{2+}-induced cortical F-actin disassembly (Sullivan et al., 2000). In activated RBL-2H3 cells calmodulin concentrated in actin-rich cell cortex (Psatha et al., 2004) where it was recruited by an interaction with the actin-binding scaffold protein IQGAP1 that sequesters Rho GTPases Rac and Cdc42 in GTP-bound state. Rho GTPases are released from IQGAP1 when IQGAP1-associated calmodulin binds Ca^{2+}. Calcium elevation therefore not only initiates the disassembly of the existing cortical actin cytoskeleton but simultaneously releases the GTP-loaded Cdc42 and Rac1 to mediate actin-based cell spreading and ruffling (Psatha et al., 2007).

ROLE OF KINASES AND PHOSPHATASES IN REGULATION OF MICROFILAMENTS IN ACTIVATED CELLS

Activation of mast cells via both FcεRI and c-Kit receptors is mediated and regulated by numerous protein tyrosine and serine/threonine kinases which transmit the downstream signals. It has been proposed that Fyn kinase, which in BMMCs participates in signal transduction from activated FcεRI, phosphorylates the non-selective Ca^{2+} channel TRPC1 (transient receptor potential cation channel subfamily C). This results in modulation of Ca^{2+} influx and F-actin depolymerization (Suzuki et al., 2010).

PI3K regulates microfilament organization in BMMCs by activation of Btk that in turn induces Rac-dependent F-actin rearrangement and enhancement of the Ca^{2+} signal. This in combination leads to synergy in mast cell chemotaxis (Kuehn et al., 2010). In BMMCs stimulated by PGE_2, PI3K activates Ser-Thr kinase, mammalian target of rapamycin (mTOR). mTOR binds to specific regulator, named rictor, to form mTORC2 complexes in association with other binding partners (Foster and Fingar, 2010). Interestingly, mTORC2 stimulated both polymerization of actin and chemotaxis, though by a mechanism independent of Ca^{2+} mobilization and Rac acctivation (Kuehn et al., 2011).

An important players are also members of p21-activated kinases (PAKs) which receive independent signals from both PI3K and Rho GTPases. PAKs induce the temporal and spatial formation of cortical actin structures similar to those regulated by Rac and Cdc42, including membrane ruffles, lamellipodia filopodia, and focal complexes (Sells et al., 2000). PAK 1-defficient BMMCs showed diminished Ca^{2+} mobilization, degranulation, and altered depolymerization of cortical F-actin in response to FcεRI stimulation. While cortical F-actin ring formed as expected in stimulated cells, subsequent fragmentation of F-actin occurred to a much smaller extent than in normal BMMCs (Allen et al., 2009). PAK 1 can modulate F-actin turnover by the well established LIMK/coffilin pathway, yet it can also phosphorylate Ser-Thr protein phosphatase 2A (PP2A) that is involved in vesicular fusion in mast cells, and transiently associate with cortical myosin II (Blank and Rivera, 2004).

New roles in regulation of microfilaments were recently ascribed tor cPKCs that are activated by DAG and by Ca^{2+}. Some data showed that cPKCβ in FcεRI-stimulated RBL-2H3 cells colocalized with F-actin in membrane-ruffled regions, and inhibition of cPKCβ resulted in the blocking of actin ruffle formation and adhesion (Yanase et al., 2011). Similar effects were observed in activated BMMCs, where furthermore migration toward SCF was suppressed (Yanase et al., 2011). In antigen-stimulated RBL-2H3 cells the cPKCβII phosphorylated non-muscle myosin heavy chain IIA, and the time course of phosphorylation correlated with degranulation (Ludowyke et al., 2006).

Recent data also confirmed the significant role of protein tyrosin phosphatases (PTPs) in regulation of microfilaments in activated mast cells. When antibody specific for oxidized (inactive) phosphatases was used and isolated membranes were examined by electron microscopy, clear colocalization of oxidized PTPs with cytoskeleton-like structures was observed in non-activated mast cells. Enhanced association of oxidized phosphatases with these structures was observed in cells activated by antigen or

by pervanadate. The same structures were labeled with phalloidin. This indicates that oxidized phosphatases are preferentially associated with actin cytoskeleton (Heneberg et al., 2010). These data suggest that actin cytoskeleton is involved in early signaling events by regulating the topography of phosphatases. Alternatively, actin could play a role in sequestering and/or scavenging irreversibly oxidized PTPs (Bugajev et al., 2010). In this connection it should be mentioned that actin is probably effective in sequestering Lyn kinase to the periphery of large FcεRI aggregates which are subsequently internalized (Wilson et al., 2000).

One of the phosphatases associated with F-actin and actin-binding protein filamin is the SH2 domain containing protein tyrosine phosphatase-1 (SHP-1). This complex associated with FcεRI coaggregated with FcγRIIB. Dissociation of actin and filamin-1 from the FcR complex caused activation of FcR-bound SHP-1 and inhibition of FcεRI-stimulated signal in RBL-2H3 cells (Lesourne et al., 2005).

As expected, phosphorylation of actin associated proteins affects microfilament dynamics in activated mast cells. An important family of such proteins are coronins. Their regulatory effects include the binding/bundling, disassembly, and inhibition of the Arp2/3 complex. Recent data showed that FcεRI-mediated phosphorylation of coronin 1a in peritoneal mast cells resulted in its relocation from the cell cortex to cytoplasm. This relocation was accompanied with reduced cortical actin stability and enhanced degranulation (Foger et al., 2011).

INTERMEDIATE FILAMENTS

Intermediate filaments provide crucial structural support in the cytoplasm and nucleus; any disturbance of intermediate filaments causes cell fragility. Intermediate filaments also play a role in determining cellular architecture, cell migration, and signal modulation. More than 70 conserved genes encode intermediate filament proteins that can self-assemble into 10-nm wide filaments. Each protein has N- and C-terminal end domains, termed "head" and "tail," respectively, surrounding the α-helical rod domain. The basic building-block for intermediate filaments is a parallel dimer formed by the winding of α-helical rods into coiled coil. Dimers then associate along their lateral surfaces with anti-parallel orientation and form apolar staggered tetramers. A tetramer represents the soluble subunit analogous to the αβ-tubulin dimer or actin monomer. However, unlike actin or tubulin, the intermediate filament subunits do not contain binding sites for nucleoside triphosphate (Coulombe and Wong, 2004). The final 10-nm filament is a helical array of tetramers. The anti-parallel orientation of tetramers means that, contrary to microtubules and microfilaments, intermediate filaments do not bear polarized unidirectional properties. Assembly and disassembly is regulated by cycles of phosphorylation and dephosphorylation. Polymerization occurs rapidly irrespective of nucleating or associated proteins. In contrast to microtubules and microfilaments, intermediate filaments do not serve as tracks for molecular motors (Kim and Coulombe, 2007).

Intermediate filament proteins are subcategorized into six types (I-VI), based on similarities in amino acid sequences and protein structure, and individual types have different cell and tissue distribution. Vimentin (type III intermediate filament protein) is the most widely distributed type that can be found in mesenchymal and connective tissue cells. Vimentin plays a significant role in supporting and anchoring the position of organelles in the cytosol. Vimentin filaments possess unique viscoelastic physical properties that render them more resistant to mechanical stress in comparison to microtubules and microfilaments (Kreis and Vale, 1999). The mechanical strength of vimentin filaments network appears to be further enhanced after interactions with microtubules (Dráberová and Dráber, 1993) and microfilaments mediated by a family of multifunctional intermediate filament-associated proteins (Green et al., 2005). Within the cells vimentin exists in multiple structural forms including mature filaments, short filaments called "squiggles," and non-filamentous precursors called "particles." The forms are intercovertible and their relative abundance is cell type- and cell cycle stage-dependent. These structures are often associated with microtubule motor proteins, and are therefore capable of translocating along microtubules (Chou et al., 2007).

INTERMEDIATE FILAMENTS IN ACTIVATED MAST CELLS

Data on the role of intermediate filaments during mast cell activation events are limited. The key building protein of intermediate filaments in mast cells is vimentin (Horny et al., 1988). It was reported that vimentin was phosporylated on serine after activation of RPMC with compound 48/80. Phosphorylation took place within 5 s of stimulation and reached its maximum in 10 s. When cells were treated with calphostin C, a specific inhibitor of protein kinase C, phosphorylation was markedly reduced. Interestingly, cells stained with anti-vimentin antibody showed filaments surrounding granules in cytoplasm; after stimulation these filaments promptly disappeared, indicating rapid depolymerization (Izushi et al., 1992). It was assumed that the disruption of intermediate filaments took place after stimulation with compound 48/80 as a consequence of vimentin phosphorylation. The absence of filaments surrounding granules facilitated then the movement of granules toward the cell membrane and degranulation (Tasaka, 1994). Mass spectrometric analysis of proteins binding to the SH2 and SH3 domains of Fyn led to identification of vimentin as the binding partner to Fyn in MC/9 mast cells. After IgE-receptor mediated stimulation, binding of vimentin to Fyn increased; this interaction occurred via binding to the SH2, but not SH3, domain of Fyn. Mast cells from vimentin-deficient mice showed an increase in mediator release and tyrosine phosphorylation of intracellular proteins including NTAL and LAT. These results suggest that vimentin association with Fyn may have a negative regulatory role in mast cell degranulation and tyrosine phosphorylation of signaling molecules induced by FcεRI stimulation (Nahm et al., 2003). Lymphocytes from vimentin-deficient mice are subject to greater deformation than lymphocytes from wild type mice, and vimentin was therefore suggested to be the primary source of lymphocyte rigidity (Brown et al., 2001). It may be that more deformable mast cells degranulate more easily following FcεRI aggregation. The observation that vimentin binds to Fyn points to a critical role for Fyn in mast cell degranulation during interaction with both cytosolic and structural proteins (Nahm et al., 2003).

CONCLUDING REMARKS

A broad range of experimental findings shows that cytoskeletal proteins in activated mast cells play an important role in propagation of the signals from stimulated cell-surface receptors FcεRI, c-Kit, or GPCR. Cytoskeletal proteins are also accountable for observed prominent changes in cell morphology, adhesion to substrates, degranulation, and migration. Some controversial results mentioned in this review probably reflect variations in the origin of mast cells, culture conditions, cell activation, methods used for visualization of cytoskeletal structures, and possibly others. The crosstalk between microtubules, microfilaments, and intermediate filaments in the course of mast cell activation is still an open question. Various cytoskeletal scaffolding proteins with numerous protein interaction domains and integrating signals from kinases/phosphatases, Rho GTPases and changes in Ca^{2+}concentration apparently affect the regulatory mechanisms of interactions between microtubules and microfilaments. The roles of posttranslational modifications of tubulins and MAPs in nucleation and modulation of microtubule dynamics in activated cells are other fields with many unsolved problems. Similarly, little is known about the role of motor proteins and their regulation in granule transport, and cytoskeleton reorganization during activation events. New innovative techniques of live cell imaging will undoubtedly play a growing role in studies on cytoskeleton dynamics in activated mast cells under physiological conditions.

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Mast cell: an emerging partner in immune interaction

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Mast cells (MCs) are currently recognized as effector cells in many settings of the immune response, including host defense, immune regulation, allergy, chronic inflammation, and autoimmune diseases. MC pleiotropic functions reflect their ability to secrete a wide spectrum of preformed or newly synthesized biologically active products with pro-inflammatory, anti-inflammatory and/or immunosuppressive properties, in response to multiple signals. Moreover, the modulation of MC effector phenotypes relies on the interaction of a wide variety of membrane molecules involved in cell–cell or cell-extracellular-matrix interaction. The delivery of co-stimulatory signals allows MC to specifically communicate with immune cells belonging to both innate and acquired immunity, as well as with non-immune tissue-specific cell types. This article reviews and discusses the evidence that MC membrane-expressed molecules play a central role in regulating MC priming and activation and in the modulation of innate and adaptive immune response not only against host injury, but also in peripheral tolerance and tumor-surveillance or -escape. The complex expression of MC surface molecules may be regarded as a measure of connectivity, with altered patterns of cell–cell interaction representing functionally distinct MC states. We will focalize our attention on roles and functions of recently discovered molecules involved in the cross-talk of MCs with other immune partners.

Keywords: mast cell, cell–cell interaction, adaptive immunity, innate immunity

INTRODUCTION: ORIGIN, DISTRIBUTION AND FUNCTIONAL HETEROGENEITY

To develop an effective immune response, the cells of the immune system are required to communicate between each other through secretion of soluble mediators and direct cell–cell interaction. Among the cells of the immune system, mast cell (MC) appears to be one of the most powerful in terms of ability to respond to multiple stimuli and to selectively release different types and amounts of mediators (reviewed in Galli et al., 2005b).

Research on MC physiopathology has changed our perception of the role that MCs play within the immune system. Indeed, their functions extend through all the stages of the immune response, ranging from shaping the response against pathogens, regulating both innate and acquired immune cell functions, to supporting regulatory cells in the maintenance of tissue-tolerance.

MCs originate from a multipotent hematopoietic progenitors in bone marrow, and then migrate through blood to tissues where they mature (Hallgren and Gurish, 2011). In mice, an hematopoietic stem cell progresses to a multipotent progenitor, a common myeloid, and a granulocyte/monocyte progenitor (Chen et al., 2005). A monopotent MC progenitor is found in bone marrow and intestine, and a common basophil/MC progenitor is also found in mouse spleen (Chen et al., 2005). After their homing in the tissues, maturation of the MC precursors is dependent on stem cell factor (SCF) expressed on the surface of fibroblasts, stromal cells, and endothelial cells (Arinobu et al., 2005).

MCs are positioned throughout the vascularized tissues and serosal cavities where they constitute one of the first cell types of the immune system able to interact with allergens and antigens (Galli et al., 2008a). Within body tissues, micro-environmental stimuli control MC phenotypic profile leading to subtype differences from a common progenitor (Moon et al., 2010). Historically, the classification of rodent MC subtypes has been based on phenotypic differences between connective tissue MCs (CTMCs), found in the skin and peritoneal cavity, and mucosal MCs (MMCs), which are mainly present in the intestinal lamina propria. There are, however, different phenotypic characteristics between these two populations and also differences in functions, histochemical staining, content of proteases, and reactivity to selected secretagogues and anti-allergic drugs. MMCs express MC protease (MMCP)-1 and -2, while CTMCs are positive for MMCP-4, -5, -6, and carboxypeptidase A. MMCs expand remarkably during T cell-dependent immune responses to certain parasites while CTMCs exhibit little or no T cell dependence (Moon et al., 2010). Human MCs also exhibit heterogeneity and are thus classified by their content of serine proteases as tryptase-only MCs (MC_T), which predominate in the alveolar septa and in the small intestinal mucosa, chymase-only MCs (MC_C), present in synovial tissue, or both tryptase- and chymase-positive MCs (MC_{TC}) which localize in skin, tonsils and small intestinal submucosa (Irani et al., 1986; Irani and Schwartz, 1994).

MAST CELL COMMUNICATION WITHIN IMMUNE SYSTEM VIA SOLUBLE MEDIATORS

MC heterogeneity depending on the tissue distribution, is reflected by their ability to react to multiple stimuli (Frossi et al., 2004) and by the numerous immunoglobulin E (IgE)-dependent and -independent activation pathways. A plethora of membrane

receptors can regulate MC activation: FcεRI and Fcγ receptors, Toll like receptors (TLRs), complement receptors, cytokine and chemokine receptors, and hormone receptors (Zhao et al., 2001; Theoharides et al., 2004; Galli et al., 2005b) as summarized in **Table 1**. Depending on the type, property, strength, and combination of the stimuli they receive, MCs secrete a diverse and wide range of biologically active products that can trigger, direct, or suppress the immune response (Frossi et al., 2004). MC soluble products, listed in **Table 2**, can be divided into two categories: (a) preformed mediators, such as histamine, proteoglycans, and neutral proteases and certain cytokines, in particular tumor necrosis factor-alpha (TNF-α), that are rapidly and instantaneously released upon MC activation; (b) newly synthesized mediators, such as cytokines, chemokines, lipid mediators, growth and angiogenic factors that start to be synthesized after MC activation (Galli et al., 2005a; Metz and Maurer, 2007). Although these products are all important in both innate and acquired immunity, the rapid release of MC mediators is crucial for the initiation of the immune response at the site of infection since they are able to modulate the immune-cell trafficking and to provide co-stimulatory signals for cell activation. In particular, focusing on rapidly released mediators, histamine is the most abundant vaso-active amine that is stored in MC granules, and it targets specific receptors on several cell types. It binds to histamine receptors on airway smooth muscle cells and on gastrointestinal cells and induces contraction and vasospasm. In addition, it has been reported that histamine is able to drive dendritic cell (DC) migration and activation (Caron et al., 2001). Among early released MC products, TNF-α is a granule-stored preformed cytokine that plays a crucial role during innate immunity as, by inducing the early influx of neutrophils, it promotes the clearance of pathogens and improves survival and morbidity (Henz et al., 2001). Serine proteases, chymase and tryptase, and the metalloprotease carboxypeptidase A are the major pre-synthesized granule components. They directly protect against parasites and venoms (carboxypeptidase A; Metz and Maurer, 2007), but also favor the expulsion of nematodes by increasing intestinal permeability (mouse MC protease-1, mMCP-1; McDermott et al., 2003), by allowing tissue remodeling, fibronectin turn-over (mMCP-4; Tchougounova et al., 2003), and induction of persistent influx of neutrophils with long lasting inflammation (mMCP-6; Huang et al., 1998).

Arachidonic acid-derived prostaglandins and leukotrienes are *de novo* synthesized metabolites of cyclooxygenase and lipooxygenase enzymes. They improve the innate response by increasing MC numbers at inflammation sites, through the recruitment of immature MCs and/or progenitors (Weller et al., 2005). MC-secreted cathelicidins reduce bacterial numbers, thus directly driving bacterial clearance (Di Nardo et al., 2003). MC-secreted compounds also contribute to the acquired immune response, serving as mediators for B and T cell recruitment and activation. MC-derived leukotriene B4 induces chemotaxis of effector $CD8^+$ T cells in the course of allergic inflammation (Ott et al., 2003), while MC-derived TNF-α is crucial in the recruitment of $CD4^+$

Table 1 | MC membrane-bound receptors.

Receptor family	Members	Reference
FcR		
FcεR	FcεRI	Kinet (1999)
FcγR	FcγRI[a], FcγRII, FcγRIII[b]	Malbec and Daëron (2007)
TLR	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10[a]	Marshal et al. (2009)
MHC	MHC class I, MHC class II	Svensson et al. (1997)
Complement receptor	CR1, CR2, CR3, CR4, CR5, C3aR, C5aR	Füreder et al. (1995)
Cytokine receptor	CD117, IL-1R, IL-3R, IL-10R, IL-12R, INFγR, TGFβR	Edling and Hallberg (2007), Moritz et al. (1998), Frossi et al. (2004)
Chemokine receptor	CCR1, CCR3, CCR4, CCR5,CCR7, CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CX3CR1	Juremalm and Nilsson, (2005)
RECEPTOR FOR ENDOGENOUS MOLECULES		
Histamine receptor	H1/H2/H3/H4 receptor	Sander et al. (2006)
Others	Endothelin-1, neurotensin, substance P, PGE2, adenosine	Galli et al. (2005b)
Adhesion molecules	ICAM-1, VCAM, VLA4, CD226 (DNAM-1), Siglec8, CD47, CD300a, CD72	Hudson et al. (2011), Collington et al. (2011), Sick et al. (2009), Bachelet et al. (2006)
CO-STIMULATORY MOLECULES		
TNF/TNFR family members	CD40L, OX40L, 4-1BB, GITR, CD153, Fas, TRAIL-R	Juremalm and Nilsson (2005), Nakae et al. (2006), Nakano et al. (2009)
B7 family member	CD28, ICOSL, PD-L1, PD-L2	
TIM family members	TIM1, TIM3	
Notch family members	Notch1, Notch2	

Some molecules have been detected only in studies on human[a] or murine[b] MCs where not indicated, molecules are expressed in both species.

Table 2 | Major MC-derived mediators.

Class	Mediators	Physiological effects
PREFORMED		
Biogenic amines	Histamine	Vasodilatation
	5-hydroxytryptamine	Leukocyte regulation, pain, vasoconstriction
Proteoglycans	Heparin, heparin sulfate	Angiogenesis, coagulation
	Chondroitin sulfate	Tissue remodeling
Proteases	Tryptase	Inflammation, pain, tissue damage, PAR activation
	Chymase	Inflammation, pain, tissue damage
	MC-CPA/Carboxypeptidase A	Enzyme degradation
	CathepsinB, C, D, E, G, L, S[b]	Pathogen killing, tissue remodeling
	MCP5/6	Pathogenesis of asthma and other allergic disorders
Lysosomial enzymes	β-hexosaminidase, β-glucuronidase, β-galactosidase, arylsulfataseA	ECM remodeling
Others	Nitric oxide synthase	NO production
	Endothelin	Sepsis
	Kinins	Inflammation, pain, vasodilatation Anti-inflammatory effects
NEWLY SYNTHESIZED		
Lipid-derived	LTB_4, LTC_4, PGD_2, PAF	Inflammation, leukocyte recruitment, endothelial adhesion, *smooth* muscle cells contraction, vascular permeability
Cytokines	IL-1α[a], IL-1β[a], IL-2[b], IL-3, IL-4, IL-5, IL-6, IL8[a], IL-9, IL-10, IL-11[a], IL-12, IL-13, IL-14[a], IL-15[a], IL-16, IL-17, IL-18[a], IL-22[b], IL-25[b], IL-33[b], MIF, TNFα, IFNα, IFNβ[b], IFNγ[b]	Inflammation, leukocyte proliferation and activation immunoregulation
Chemokines	CCL1, CCL2, CCL3[a,b], CCL4[a] CCL5[a], CCL7[a,b], CCL8[a], CCL11[a], CCL13[a], CCL16[a], CCL17, CCL19[a], CCL20[a], CCL22a,[b], CCL25[b] CXCL1[a], CXCL2, CXCL3[a], CXCL4, CXCL5, CXCL8[a], CXCL10[a], CX3CL	Leukocyte chemotaxis
Growth factors	TGFβ, SCF[a], G-CSF, M-CSF, GM-CSF, VEGF, NGFβ, LIF[a], bFGF	Growth of various cell types
Antimicrobic species	Antimicrobial peptides, NO, superoxide, ROS	Pathogen killing

Some mediators have been detected only in studies on human [a] or murine [b] MCs or not investigated[n] where not indicated molecules are expressed in both species.
General references: Galli et al. (2005a), Metz and Maurer (2007).

T cells to draining lymph nodes, during *Escherichia coli* infection (McLachlan et al., 2003). In addition, a TNF-α-dependent effect on Langerhans cells that migrate from skin to draining lymph nodes following response to bacterial peptidoglycan has been reported (Jawdat et al., 2004).

The anti-inflammatory properties of MCs were explored *in vivo*, providing evidence about MC ability to suppress the development and magnitude of the adaptive immune response (reviewed in Galli et al., 2008a). Indeed, MC-derived histamine seems to be responsible of the systemic immunosuppression of contact hypersensitivity (CHS) responses achieved by the ultraviolet B (UVB) irradiation of the skin (Hart et al., 1998), while MC-derived IL-10 limits the response to allergic contact dermatitis (Grimbaldeston et al., 2007). MC-derived IL-10 has been implicated as a mechanism of negative immune-modulatory effects following *Anopheles* mosquito bites or in peripheral tolerance to skin allograft (Depinay et al., 2006; Lu et al., 2006), but other soluble or surface molecules might be responsible for MC negative immunomodulatory functions. The mechanisms controlling the immunosuppressive function of MCs are under investigation and might be considered for pharmacological intervention to modulate the immune system in inflammatory diseases.

PATTERN OF MC MEMBRANE-BOUND MOLECULES REGULATING IMMUNOLOGICAL EFFECTOR FUNCTIONS

Mast cells express a broad array of cell surface receptors and ligands involved in cell–cell and cell-extracellular-matrix adhesion, which mediate the delivery of co-stimulatory signals that empower these cells to interact with different immune- and non-immune cells. These interactions are often bi-directional, fulfilling mutually regulatory, and/or modulatory roles, including influences on several cellular processes, such as proliferation and gene transcription. Accordingly, MC effector function plasticity might depend not only on the activatory/inhibitory signals and on the specific released mediators, but also on the secondary, co-stimulatory signals that they receive from their cellular partners in the microenvironment. Thus, MCs specialize in establishing reliable, wideband communication with other cells, orchestrating the overall immune response (Bachelet and Levi-Schaffer, 2007).

Here, we aim to describe the recent advances in contact-mediated co-stimulatory pathways connecting MC with innate and acquired immune cells. The molecules that mediate the cross-talk between MCs and their cell partners are all listed in **Table 3**.

MC AND INNATE IMMUNE CELLS

MCs and dendritic cells

The close apposition of MCs and DCs in sub-epithelial areas as sentinel of invading antigen, has led investigators to propose their potential functional partnership in modulation of the immune responses to environmental changes (Mazzoni et al., 2006; Otsuka et al., 2011). DCs do not only represent a single uniform population but display a considerable degree of heterogeneity which complicates the network of interactions with MCs subtypes (Shortman and Liu, 2002). MCs express several molecules (TNF-α, histamine, PGD2, chemokines) that might affect DC function in peripheral inflamed tissues. Both human and mouse IgE-activated MCs have been widely implicated in the process of DC mobilization from tissue to secondary lymphoid organs (Jawdat et al., 2004; Suto et al., 2006; Dawicki et al., 2010), DC maturation (Skokos et al., 2003; Kitawaki et al., 2006), and DC capacity to promote T cell responses (Kitawaki et al., 2006; Leonard et al., 2006; Mazzoni et al., 2006; Dudeck et al., 2011).

To date, while exchange of soluble mediators between MCs and DCs has been well characterized, data regarding MCs-DCs direct cross-talk are very scarce. Nonetheless, some clues are been unveiled.

In an *in vitro* cultured human system, a combinatorial effect of various factors which are able to activate human cord blood-derived MCs, including those acting in a cell contact-dependent fashion, are required for the optimal induction of Th2-promoting human monocyte-derived DCs (Kitawaki et al., 2006). Moreover, it has been shown that murine peritoneal MCs (PCMCs) can undergo a dynamic interaction with immature DCs, inducing DC maturation and the release of the T cell modulating cytokines IFN-γ, IL-2, IL-6, and TGF-β. Such PCMCs-primed DCs subsequently induced T cell proliferation and Th1 and Th17 responses (Dudeck et al., 2011). Studies in mice report that bone marrow-derived MCs

Table 3 | MC physical interactions with other immune cells.

Cell types	MC molecule	Partner molecule	Effect on MC	Effect on partner cell	Reference
MC-DC	ICAM-1	LFA-1	↑ Ca^{++} influx	↑ Maturation and chemotaxis	Otsuka et al. (2011)
MC-MDSC	n.i.	n.i.	↑ Recruitment and survival	↑ Migration and suppression activity	Yang et al. (2010)
MC-NK	CXCL8	CXCR1	n.i.	↑ Recruitment	Burke et al. (2008)
	OX40L	OX40	n.i.	IFNγ production	Vosskuhl et al. (2010)
MC-Eos	CD226	CD112	↑ Degranulation	n.i.	Bachelet et al. (2006)
	CD48	2B.4	n.i.	↑Survival	Elishmereni et al. (2010)
	n.i.	n.i.	Transfer of tryptase	↑EPO and cytokine release, transfer of EPO	Minai-Fleminger et al. (2010)
MC-$CD4^{+}$T	ICAM-1	LAF-1	↑ Degranulation and cytokine release	↑ Activation and proliferation	Inamura et al. (1998), Mekori and Metcalfe (1999)
	ICAM-1	LFA-1	Adhesion to endothelial cell	n.i.	Brill et al. (2004)
	LTβR	LTβR ligand	↑ Cytokine release	n.i.	Stopfer et al. (2004)
	OX40L	OX40	n.i.	↑ Activation and proliferation	Frandji et al. (1993), Fox et al. (1994)
	MHC-II	TCR	n.i.	Cell activation	Kashiwakura et al. (2004)
	ICOSL	ICOS	n.i.	Switch to IL-10 regulatory T	Gaudenzio et al. (2009), Kambayashi et al. (2009), Valitutti and Espinosa (2010), Nie et al. (2011)
MC-$CD^{+}8$	MHC-I	TCR	n.i.	Cell activation	Malaviya et al. (1996)
	MHC-I	TCR	↑ Expression of co-stimulatory molecules and degranulation	Cell activation	Stelekati et al. (2009)
MC + Treg	OX40L	OX40	↓ Degranulation	↓ Suppressive activity, conversion to Th17	Gri et al. (2008), Piconese et al. (2009)
	TGFβR	TGFβ membrane-bound	↓ Degranulation, ↑ IL-6 production	↓ Suppressive activity	Ganeshan and Bryce (2012)
MC + B	CD40L	CD40	n.i.	↑ Proliferation and Ig switch	Gauchat et al. (1993), Merluzzi et al. (2010)

n.i., not investigated.

(BMMCs) promote the maturation and chemotactic activity of bone marrow DCs through direct cell–cell interaction during the sensitization phase of CHS response. BMMCs and bone marrow derived immature DCs interact throughout intracellular adhesion molecule (ICAM)-1 and lymphocyte function-associated antigen (LFA-1) enhancing DC expression of the CD40, CD80, CD86, and CCR7 co-stimulatory molecules, thus promoting maturation and chemotaxis of DCs (Otsuka et al., 2011). It is possible to argue that DCs make use of MC-induced active LFA-1 to control the contact duration with naive T cells and to promote T cell priming (Balkow et al., 2010). On the other hand, co-cultures of stimulated bone marrow derived DCs with BMMCs increases calcium influx and up-regulate membrane-bound TNF-α (Otsuka et al., 2011).

MCs and natural killer cells

Concerning the cellular interactions that play a role in innate immune defense, emerging evidences show MC-dependent natural killer (NK) cell recruitment and activation. NK cells are granular cytotoxic and circulating lymphocytes involved in the clearance of transformed and pathogen-infected cells. As a part of the innate immune system, their recruitment to the site of infection is mediated by a large spectrum of chemokines which bind to the chemokine receptors, CCR2, CCR5 and CXCR3 on NK cells. Activated MCs can induce NK cell accumulation in different disease models. For instance, immune surveillance by MCs is important for NK cell recruitment and viral clearance during Dengue infection (St John et al., 2011). Human cord blood-derived MCs stimulated with virus-associated TLR3 agonist can recruit human NK via the CXCL8 and CXCR1 axis, underlining MC role as sentinel cell during early viral infections (Burke et al., 2008). Lipopolysaccharide (LPS)-activated BMMCs induce cell contact-dependent IFN-γ secretion by NK cells, without affecting cell-mediated cytotoxicity. Cellular interaction is partly mediated by OX40L expression on MCs (Vosskuhl et al., 2010). In the cited work, authors underline that different MC signals of activation confer different results in terms of NK activation. In fact, in addition to LPS, stimulation of MCs via TLR3 and TLR9, but not with IgE/antigen, amplifies IFN-γ secretion by NK cells (Vosskuhl et al., 2010). Similarly, in a model of hepatocarcinoma, MC protumoral role is associated with reduction of NK cell number and activation. This effect was due to the fact that, in the tumor micro environment, SCF-activated MCs release adenosine that inhibit production of IFN-γ by NK cells (Huang et al., 2008). Enhanced CCL3-mediated recruitment of NK cells is instead observed in a orthotopic melanoma model in which TLR2-activated MCs exert anticancer properties by secreting large amounts of this chemokine (Oldford et al., 2010).

MCs and eosinophils

Mast cells and eosinophils (Eos) co-exist in tissues during the late and chronic phases of allergic reaction where the intracellular events following IgE/Ag-induced MC activation lead to the release of pro-inflammatory mediators, which cause the immediate, early-phase of the allergic process within minutes of allergen exposure (Williams and Galli, 2000), and induce the recruitment of inflammatory cells, i.e., macrophages, T cells, Eos, basophils, and perhaps invariant NK T cells (Galli et al., 2008b). These cells, and mainly the Eos, cause the onset of the late phase of allergic response that usually occurs a few hours later (Metz et al., 2007). Nevertheless, a clear cut interplay between MCs and Eos has been proven not only in allergic inflammatory tissues (Minai-Fleminger and Levi-Schaffer, 2009; Wong et al., 2009), but also in gastric carcinoma (Caruso et al., 2007), chronic gastritis (Piazuelo et al., 2008), Crohn's disease, and Ascaris infection (Beil et al., 2002), leading to new perspectives of the current research in this area.

Eos and MCs may mutually influence each other functions by a variety of paracrine and receptor/ligand-dependent signals. In this context, some surface molecules are potential candidates to mediate MC-Eos physical contact. A considerable advance in understanding MC-Eos interaction in a human system was made by Levi-Schaffer and coworkers. CD48 and 2B4 expressed by human cord blood-derived MCs and peripheral Eos, respectively mediate the MC-Eos physical interface as a co-stimulatory signaling switch, inducing effect on Eos viability and activating Eos to release eosinophil peroxidase, IFN-γ and IL-4 (Elishmereni et al., 2010). Similarly, evidence for a role of CD226/CD112 interaction in Eos-dependent enhancement of IgE-induced MCs activation has been described (Bachelet et al., 2006). Other ligand-receptor interactions between MCs and Eos seem to be mediated through LFA-1 and ICAM-1. This pathway can be activated upon MC degranulation and results in the recruitment of eosinophils at the site of inflammation (Elishmereni et al., 2010). Moreover, by transmission electron microscopy it has been possible to demonstrate that human peripheral blood Eos and cord-blood-derived MC functionally adhere to each other as Eos peroxidase (EPO) is transferred from Eos to MCs and tryptase from MCs to Eos, thus indicating that MCs and Eos show signs of reciprocal activation (Minai-Fleminger et al., 2010).

MCs and neutrophils

Polymorphonuclear neutrophils (PMNs) constitute the most abundant leukocyte population in the peripheral blood of humans, make a highly significant contribution to the host defense, and are particularly well studied in the context of bacterial infection. However, PMN are more versatile as there is increasing evidence for their participation in acute and chronic inflammatory processes, in the regulation of the immune response, in angiogenesis, and in the interaction with tumors (Fridlender et al., 2009; Mantovani et al., 2011). PMNs have emerged as an important component of effector and regulatory circuits in the innate and adaptive immune systems. In contrast to the traditional view of these cells as short-lived effectors, evidence now indicates that they have diverse functions. By responding to tissue- and immune cell-derived signals and by undergoing polarization, PMNs are reminiscent of macrophages (Fridlender et al., 2009; Biswas and Mantovani, 2010). PMNs engage in bi-directional interactions with diverse components of both the innate and adaptive immune systems and can differentially influence the response depending on the pathological context. With the advent of MC-deficient mice and the ability to selectively reconstitute their deficiency it has been possible to show that MCs are critical for the PMN activation. Thus, in a model of immune complex-mediated peritonitis, the rapid recruitment of PMNs turns out to be initiated by LT produced by MCs, which are strategically located at the host-environment

interface (Ramos et al., 1990, 1991). In addition, in the same model, the recruitment of PMN at late phase was dependent also on MCs, and on MCs-released TNF-α (Zhang et al., 1992). The unique ability of MCs to store and immediately release TNF-α on demand, and subsequently as newly synthesized inflammatory molecule, is essential for the rapid onset and for the sustaining of inflammatory reactions (Wershil et al., 1991). A cornerstone in this context was the observation that MCs and MC-derived TNF-α initiate the life-saving inflammatory response rapidly upon encountering microbes and microbial constituents through the influx of neutrophils in mouse models for acute bacterial infections (Echtenacher et al., 1996). In murine infectious peritonitis it has been published that, besides TNF-α, several other MC-derived molecules have a role in the recruitment of PMNs. In fact, MC-derived LT (Ramos et al., 1991), mouse MC protease 6 (mMCP-6; Caughey, 2007), and the chemokine MIP-2 (CXCL2; Wang and Thorlacius, 2005) are critical for a rapid and protective influx of PMNs. The available data suggest that mMCP-6 triggers the release of MIP-2 from endothelial cells ("activation" of endothelial cells), which in turn enhances the release of MC-derived TNF-α, followed by sustained secretion of LT.

However, in this contest in which a clear functional interaction between MC and PMNs has been established, receptors-ligand pair that might physically mediate the cross-talk between these two cell populations have not yet been described.

MCs and myeloid derived suppressor cells

A complex network of cellular interactions characterizes tumor microenvironment with the presence of immune-suppressive and pro-inflammatory cells. MCs are known actors in cancer setting thanks to their ability to directly influence tumor growth, angiogenesis, and tissue remodeling and to exert an indirect function by immune-modulating cancer microenvironment. A closed loop amongst MCs and myeloid derived suppressor cells (MDSCs), also involving regulatory T (Treg) cells, has been recently described in murine hepatocarcinoma tumor microenvironment. MCs promote the migration and suppressor function of tumor MDSCs by CCL-2 and 5-lipoxygenase release, further exacerbating tumor inflammatory microenvironment. Indeed, MCs stimulate MDSCs to secrete the pro-inflammatory cytokine IL-17 which stimulate Treg cells to release IL-9 which in turn, strengthen the survival and protumoral effect of MCs (Yang et al., 2010; Cheon et al., 2011).

These are preliminary studies that disclose a novel relationship between MDSCs, MCs, and Treg cells. Further analysis will determine whether these cells physically interact through co-stimulatory molecules.

MC AND ADAPTIVE IMMUNE CELL

MCs and effector T cells

The close physical apposition between MC and T cell has been observed during T cell-mediated inflammatory processes (Mekori and Metcalfe, 1999), such as cutaneous delayed-type hypersensitivity (Dvorak et al., 1976; Waldorf et al., 1991), sarcoidosis (Bjermer et al., 1987), and in chronic inflammatory processes associated with the pathology of inflammatory bowel disease and rheumatoid arthritis (Marsh and Hinde, 1985; Malone et al., 1986). Moreover, morphological studies have revealed that MCs reside in close physical proximity to T cells in inflamed allergic tissues and at sites of parasitic infections (Friedman and Kaliner, 1985; Smith and Weis, 1996).

Some of such influences have been attributed to the biological effects of a wide range of soluble mediators; however increasing amounts of literature documents recognize the importance of intercellular communication involving the binding of cell surface molecules.

Early studies demonstrated that intercellular contacts between MC and T cell lines are able to activate MC transcription machinery (Oh and Metcalfe, 1996). Adhesion of HMC-1 human MC line, or murine BMMCs, to activated T lymphocytes induces MC degranulation and TNF-α production (Bhattacharyya et al., 1998). Moreover, the MC-T cell cross-talk results in the release of matrix metalloproteinase (MMP)-9 and the tissue inhibitor of metalloproteinase 1 from HMC-1 human MCs or from mature peripheral blood-derived human MCs. This effect, as well as the secretion of β-hexosaminidase and several inflammatory cytokines (TNF-α, IL-4, and IL-6), is mediated by a direct contact of activated, but not resting, T cell membranes with MCs (Baram et al., 2001). In accordance with these findings, a recent study revealed that activated T cell microparticles, small membrane-bound structures released from cells during activation or apoptosis, are able to induce the production of soluble mediators from LAD2 human cell line and human cord blood-derived MC cultures. By releasing microparticles, T cells may convey surface molecules and activate distant MCs within the same inflammatory site (Shefler et al., 2010). Other heterotypic adhesion-induced effects on MC activation have been described. The proximity of activated T lymphocytes to HMC-1 promotes MC adhesion to the receptor of endothelial cells as well as to the extracellular matrix ligands (Brill et al., 2004).

The adhesion pathway mediated by LFA-1and its ligand ICAM-1 induced FcεRI-dependent murine BMMC degranulation after heterotypic aggregation with activated T cells and was the first membrane-bound pathway involved in MC/T cell cross-talk to be described (Inamura et al., 1998). In addition, lymphotoxin-β receptor (LTβR) expressed on murine BMMCs can be triggered by LTβR ligands expressed by T cell lines and transduces a co-stimulatory signal leading to the release of cytokines (IL-4, IL-6, TNF-α) and chemokines (CXCL2 and CCL5) from ionomycin-activated BMMCs (Stopfer et al., 2004). Moreover, the engagement of OX40 on activated $CD4^+$ T cells by OX40L-expressing MCs, together with the secretion of soluble MC-derived TNF-α, costimulates proliferation and cytokine production from activated $CD4^+$ T cells (Nakae et al., 2006). Similar results were also established in a culture system of human tonsillar MCs and human T cells which confirmed the enhancement of T cell proliferation upon direct OX40/OX40L engagement demonstrating the presence of a bi-directional cellular cross-talk among these cell types (Kashiwakura et al., 2004). The existence of functional MC-T cells interaction also arises from the observation that murine BMMCs could present antigenic peptides to T cell lines and $CD4^+$ T cell hybridoma (Frandji et al., 1995, 1996). MHC-II-dependent antigen presentation to $CD4^+$ T cells by MCs was also demonstrated in rat and human cell systems (Fox et al., 1994; Poncet et al., 1999) reinforcing the concept that MCs can serve as unconventional antigen presenting cells for T lymphocytes (Valitutti and Espinosa,

2010). More recently, it has been proposed the possibility that MCs can be primed to acquire APC phenotype. To date, inducible expression of MHC-II molecules, MHC-II associated molecules as well as OX40L and PD-L1, by murine BMMCs, spleen-derived MCs, and peritoneal MCs has been reported in response to various *in vitro* treatments (Gaudenzio et al., 2009; Kambayashi et al., 2009; Nakano et al., 2009). It has been shown that Notch signaling induces MHC-II and OX40L expression and can thus elicit the commitment of BMMC to an APC population, which promotes the differentiation of naive $CD4^+$ T cells toward conventional Th2 cells (Nakano et al., 2009). It also appears that MHC-II expression grants MCs the ability to effectively support T cell proliferation and effector functions and causes expansion of Treg cells (Kambayashi et al., 2009). In addition, a recent work provides experimental morphological evidence of direct antigen presentation by peritoneal cell-derived MCs and freshly isolated peritoneal MCs at a single-cell level, eliciting functional responses in effector T cells, but not in their naïve counterparts (Gaudenzio et al., 2009). Similarly, MCs are capable of inducing antigen-specific $CD8^+$ T cell responses *in vitro* and *in vivo*. Murine BMMCs can, in fact, process Ag from phagocytosed bacteria for presentation via MHC class I molecules to T cells (Malaviya et al., 1996). Moreover, MHC class I dependent cross presentation of BMMCs and peritoneal MCs to $CD8^+$ T cells was recently shown to increase $CD8^+$ T cell proliferation, cytotoxic potential, and degranulation. In turn, $CD8^+$ T cells induce MHC class I and 4-1BB expression on BMMCs as well as the secretion of osteopontin (Stelekati et al., 2009).

MCs and natural and inducible regulatory T cells

In the complex network of immune interactions, the amount of information available on the functional interaction between MCs and immunoregulatory cells is going to increase. MCs and natural $CD25^+$ $Foxp3^+$ Treg cells have been demonstrated to reside in close proximity in secondary lymphoid tissues as well as in mucosal tissues (Vliagoftis and Befus, 2005; Gri et al., 2008) and to influence each others' function. Indeed, activated Treg cells caused a reduction in the expression of FcεRI on murine BMMCs by contact-dependent mechanism and production of soluble factors such as TGF-β and IL-10 (Kashyap et al., 2008). Treg cells can hinder BMMC degranulation and immediate hypersensitivity response through the engagement of OX40L on MCs (Gri et al., 2008). Treg cell-mediated inhibition of MC function is regulated at a single-cell level and is not restricted to BMMCs, but is a common feature of murine PCMCs and human LAD2 MC line (Frossi et al., 2011).

A recent study confirmed that co-culture of Treg cells with murine BMMC suppresses degranulation but primes MCs for production of IL-6 via a contact-dependent surface-bound TGF-β mechanism (Ganeshan and Bryce, 2012). Interestingly, in a model of colorectal cancer, highly suppressive Treg cells lose the ability to suppress human LAD2 MC degranulation (Blatner et al., 2010), suggesting that a complex interaction between MCs and Tregs within tumor microenvironment exists, although the mechanism behind these events has not been yet discovered. Conversely, MC activation breaks peripheral tolerance. Direct cell–cell contact, dependent on OX40/OX40L interaction, and T cell-derived IL-6 promotes Th17 skewing of Treg cells with loss of both Foxp3 expression and T cell suppressive properties *in vitro*. Activated MCs, Tregs, and Th17 cells display tight spatial interactions in lymph nodes hosting T cell priming in experimental autoimmune encephalomyelitis further supporting the occurrence of an MC-mediated inhibition of Treg suppression in the establishment of Th17-mediated inflammatory responses (Piconese et al., 2009).

Under certain conditions such as in inflammation and immune reactions, increasing expression of ICOSL might contribute to the regulatory role of MCs. Indeed, *in vitro* experiments and the *in vivo* model of neutrophilic airway inflammation, allowed the identification of an intimate link between LPS-stimulated murine BMMCs, which upregulate ICOSL surface expression, and the generation of IL-10 producing inducible regulatory $CD4^+$ T cell with inhibitory ability on effector T cells function. Indeed, ICOSL-deficient BMMCs are not able to sustain IL-10 producing T cell activation (Nie et al., 2011).

MCs and B cells

Mast cells produce several cytokines, such as IL-4, IL-5, IL-6, and IL-13, that are known to regulate, directly or in combination with other factors, B cell development and function. Moreover, the CD40L co-stimulatory molecule is expressed on the surface of activated-BMMCs, skin MCs, and MCs under allergic inflammatory conditions (Gauchat et al., 1993; Pawankar et al., 1997). These data further support the existence of a functional cross-talk between these two cell types. The first evidence of an effective MC-B cell cross-talk, mediated by the physical interaction through the CD40L:CD40 axis, was reported by Gauchat and coworkers. They showed that CD40L was expressed on both freshly purified human lung MCs and on the human cell line HMC-1 and further demonstrated that these MCs can interact with B cells to induce the production of IgE, in the presence of IL-4 and in absence of T cells (Gauchat et al., 1993). Furthermore, the role of the CD40-CD40L axis in the induction of IgE production by B cells was also observed in perennial allergic rhinitis (PAR), an IgE-mediated atopic disease. Nasal MCs (NMCs) from patients with PAR displayed significantly higher expression levels of FcεRI, CD40L, IL-4, and IL-13 compared to NMCs from patients with chronic infective rhinitis (CIR). The essential role of CD40L in this allergic disease context was further substantiated by the finding that the IgE production was inhibited by anti-CD40L mAb (Pawankar et al., 1997). The group of Mécheri was the first to show that unstimulated BMMCs were able to induce resting B cells to proliferate and to become IgM-producing cells. In this case, B cell activation was mediated by MC-derived factors and contact between these two cell types seemed not to be required (Tkaczyk et al., 1996). Some years later, the same research group reported that membrane vesicles, released by the MC cytoplasmic granules and termed exosomes, were responsible of MC-driven B cell proliferation and activation. Interestingly, they showed that important co-stimulatory molecules, such as MHC-II, CD86, CD40, CD40L, LFA-1, and ICAM-1, were associated with exosomes (Skokos et al., 2002).

Only recently the study of the specific role of MCs in B cell growth and differentiation has been investigated more in detail. Merluzzi and coworkers proved that both resting and activated

MCs were able to induce a significant inhibition of cell death and an increase in proliferation of naïve B cells. Such proliferation was further enhanced in activated B cells. This effect required cell–cell contact and MC-derived IL-6. Activated MCs were shown to regulate CD40 surface expression on unstimulated B cells and the interaction between CD40 and CD40L on MCs, together with MC-derived cytokines, were involved in the differentiation of B cells into $CD138^+$ plasma cells and in selective IgA secretion. These data were corroborated by *in vivo* evidence of infiltrating MCs in close contact with IgA-expressing plasma cells within inflamed tissues (Merluzzi et al., 2010).

CONCLUDING REMARKS

In the last few years, our perception of MCs function has dramatically changed. In fact, there has been mounting evidence that the function of these cells is not limited to acting as first line of defense against invading pathogens or as effector cells in allergy, but is extended to perform additional and unexpected activities in strict collaboration with adaptive immune and other non-immune cells. Thus, MCs together with other innate and adaptive immune cells orchestrate complex functional programs to promote host defense, to control the development of self-tolerance, and to avoid autoimmunity. In this context, the gene expression pattern, the phenotype, as well as MC function must rapidly change in a coordinate, time-dependent manner in response to micro-environmental soluble and cellular signals. In view of their extensive assortment of membrane receptors able to mediate delivery of co-stimulatory signals, of molecules involved in cell-extracellular-matrix adhesion and in cell–cell contacts and of soluble pro- and anti-inflammatory mediators, MC may profoundly influence the development, intensity, and duration of adaptive immune responses that ultimately serve for host defense, allergy, and autoimmunity. Considering the continuously emerging findings in the field, it is predictable that in the next years we will assist to the discovery of additional, unsuspected biological features that MCs possess.

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The SNARE machinery in mast cell secretion

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Mast cells are known as inflammatory cells which exert their functions in allergic and anaphylactic reactions by secretion of numerous inflammatory mediators. During an allergic response, the high-affinity IgE receptor, FcεRI, becomes cross-linked by receptor-bound IgE and antigen resulting in immediate release of pre-synthesized mediators – stored in granules – as well as in *de novo* synthesis of various mediators like cytokines and chemokines. Soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNARE) proteins were found to play a central role in regulating membrane fusion events during exocytosis. In addition, several accessory regulators like Munc13, Munc18, Rab GTPases, secretory carrier membrane proteins, complexins, or synaptotagmins were found to be involved in membrane fusion. In this review we summarize our current knowledge about the SNARE machinery and its mechanism of action in mast cell secretion.

Keywords: mast cell, exocytosis, SNARE proteins

INTRODUCTION

Mast cells are tissue-localized cells that upon activation release a whole variety of inflammatory mediators (Blank and Rivera, 2004; Rivera and Gilfillan, 2006). When released, their role is to protect the body against infectious agents, injury or stress, however, inappropriate or chronic production may also have harmful consequences and engender inflammatory diseases (Bischoff, 2007; Kalesnikoff and Galli, 2008; Abraham and St John, 2010). Mast cells are best-known for the role they play in allergic diseases after stimulation through IgE bound to high-affinity IgE receptors. However, recent years have made clear their general role both in protective and disease-promoting inflammatory responses that involve stimulation through a wide array of surface-expressed receptors including IgG Fc receptors, different types of G protein-coupled receptors, Toll-like receptors, etc. (Bischoff, 2007; Kalesnikoff and Galli, 2008; Abraham and St John, 2010; Beghdadi et al., 2011). While initial therapeutic strategies aiming to restrict mast cell activation largely focused on blocking the activation of the IgE receptor and its early signaling events, targeting the late signaling steps has become a suitable alternative strategy. The latter approach would block the consequences of activation by many different receptor types converging into the same secretory pathways.

With the exception of lipid-derived mediators that are synthesized at the plasma membrane, mast cells secrete their mediators either by a process called degranulation from sources pre-stored in cytoplasmic granules including for example histamine and certain mast cell specific proteases or through carrier vesicles emanating from the Golgi including a large array of cytokines/chemokines (Blank and Rivera, 2004; Sagi-Eisenberg, 2007). Considerable advances have been made in the understanding of the molecular machinery involved in vesicular secretion. In this review we provide an overview of the current knowledge on the mechanisms of mast cell exocytosis as well as our current ideas about its mechanisms of regulation.

CHARACTERISTICS OF MAST CELL EXOCYTOSIS

Transport of vesicles and exocytosis of mediators are cellular processes that occur in all eukaryotic cells. Newly synthesized mediators must be translocated into the endoplasmic reticulum and then transported to the Golgi apparatus, where secretory cargoes are sorted into a variety of transport carriers for delivery to their final destinations (Glick and Nakano, 2009). Some secretory cells including mast cells are capable of regulated exocytosis (Lacy and Stow, 2011). They store a wide range of factors and immune mediators in pre-formed secretory granules (SG). Like in other hematopoietic cells with SG these are dual-function organelles containing lysosomal hydrolases such as β-hexosaminidase and cathepsin-D as found in all lysosomes as well as specific secretory inflammatory products (Stinchcombe and Griffiths, 2007). They are therefore often called secretory lysosomes to underline the close connection between endosomal/lysosomal and secretory compartments. Functionally, secretory lysosomes are unusual in

that they serve both as a degradative and as a secretory compartment (Blott and Griffiths, 2002). Upon activation mast cells release a high amount of granule contents (Blank and Rivera, 2004). Membrane fusion, necessary for the stimulus-coupled release of granule contents, requires that lipid molecules leave their bilayer orientation to merge two lipid bilayers (Cohen and Melikyan, 2004). In neurons, where each vesicle interacts independently with the plasma membrane, exocytosis is coupled to rapid endocytosis and regeneration of SG within milliseconds until seconds. In contrast, mast cells are capable of releasing high amounts of their granular content in response to a single stimulatory event, a process called degranulation, and the regeneration of a granule can take up to 72 h (Galli et al., 1984; Gandhi and Stevens, 2003; Blank and Rivera, 2004). A resting human intestinal mast cell and a mast cell following activation induced degranulation are shown in **Figure 1**. Unlike neurons, mast cells SG can form channels by fusing with each other. This so-called compound exocytosis can either occur in a sequential or in a multivesicular manner. In sequential exocytosis, vesicles initially fuse with the plasma membrane followed by the fusion of underlying next vesicles with the first vesicle. In multivesicular exocytosis, vesicles fuse with each other before interacting with the plasma membrane (Alvarez de Toledo and Fernandez, 1990). Compound exocytosis enables mast cells to discharge their contents very efficiently (Pickett and Edwardson, 2006). In response to some stimuli, mast cells show also so-called piecemeal degranulation characterized by gradual loss of granule contents without detectable granule fusion. Thus, piecemeal degranulation allows discharge of discrete packets of granule-associated components without granule exocytosis (Dvorak, 2005). While mast cell mediator release occurs typically, although not exclusively, multi-directionally, other cell types of the immune system such as cytotoxic T cells or natural killer (NK) cells use secretory lysosomes to deliver proteins involved in their effector functions at the immune synapse in a uni-directional, polarized manner. Mast cells also secrete a large variety of inflammatory cytokines, chemokines, and growth factors. Although some of them have been found to be stored in secretory lysosomes secretion of newly synthesized products occurs independently of SG exocytosis. Furthermore, some stimuli like TLR4 ligands or IL-1 induced secretion in the absence of SG release (Kandere-Grzybowska et al., 2003; Qiao et al., 2006).

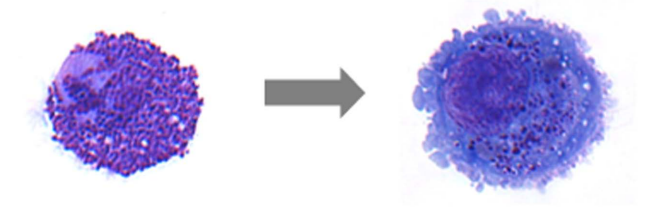

FIGURE 1 | May–Grünwald/Giemsa stain of a resting human intestinal mast cell and a mast cell following activation induced degranulation. Note the increase in size and loss of granule staining.

SNARE PROTEINS IN DEGRANULATION

The fusion between vesicles or the plasma membrane is not a spontaneous event. It requires a specific set of proteins called *Soluble N-ethylmaleimide-sensitive factor attachment protein receptors* (SNAREs) that are highly conserved in all eukaryotes (Sudhof and Rothman, 2009; Sudhof and Rizo, 2011). They were initially discovered by several independent approaches involving yeast genetics and biochemical purification procedures from synaptic membranes and by the ability to bind soluble N-ethylmaleimide-sensitive factor (NSF)-attachment proteins, which are adapters that connect the fusion machinery to the NSF ATPase (Novick et al., 1980; Bennett and Scheller, 1993; Sollner et al., 1993). The SNARE machinery of membrane fusion involves different sets of proteins that lie on opposing membranes. They enable fusion by forming a highly stable tetrameric trans-SNARE complex through four conserved 60–70 aa SNARE motifs (Sutton et al., 1998). Dissociation of this complex is the energy-requiring step in fusion and is mediated by the NSF ATPase (Hanson et al., 1997). A typical trans-SNARE complex at the plasma membrane includes a vesicular SNARE (v-SNARE) such as vesicle associated membrane protein (VAMP) that pairs with two target membrane SNAREs (t-SNAREs) such as a Syntaxin (STX) molecule and synaptosome-associated protein of 23 (ubiquitous) or 25 (neuronal) kDa (SNAP-23/25) containing two SNARE motifs (Sutton et al., 1998). To take into account that v-SNAREs can also be found on the target membrane, for example in the case of homotypic vesicle fusion, SNAREs have also been classified structurally into R-SNAREs (corresponding with few exceptions to v-SNAREs) based on a central R residue in the 0 layer of the classical four-helix-bundle of the SNARE complex and Q-SNAREs with a central Q residue (Hong, 2005). Trans-SNARE complex, generally consists of either one v-SNARE and two or three t-SNAREs or one R-SNARE and two or three Q-SNAREs. **Figure 2A** illustrates SNARE complex formation catalyzing granule fusion in mast cells and **Figure 2B** shows the domain structure of these SNAREs and potential phosphorylation sites.

Mast cells express a wide array of SNAREs albeit their localization may differ between different cell types and species. To date, described SNARE proteins in mast cells include the t-SNAREs SNAP-23 as well as STX2, 3, 4, and 6. VAMP family protein members include VAMP2, 3, 4, 7, and 8 (Sander et al., 2008; Benhamou and Blank, 2010). Their functional implication in secretory mechanisms has been partially explored, but not in all cases precise colocalization studies with known marker proteins of mast cell compartments have been performed. The first study demonstrating SNARE-mediated contribution to mast cell degranulation was published in 1998 by the group of D. Castle (Guo et al., 1998). They showed that introduction of antibodies directed to SNAP-23 into permeabilized rat peritoneal mast cells inhibited exocytosis independent of whether it was stimulated through GTPγS or calcium. During exocytosis plasma membrane-localized SNAP-23 relocated into the interior of the cell along degranulation channels in agreement with a compound mode of exocytosis. In another study overexpression of SNAP-23, but not of a derived VAMP-binding mutant, enhanced mast cell exocytosis (Vaidyanathan et al., 2001). Concerning STX family members it was reported in the RBL mast cell line that STX4

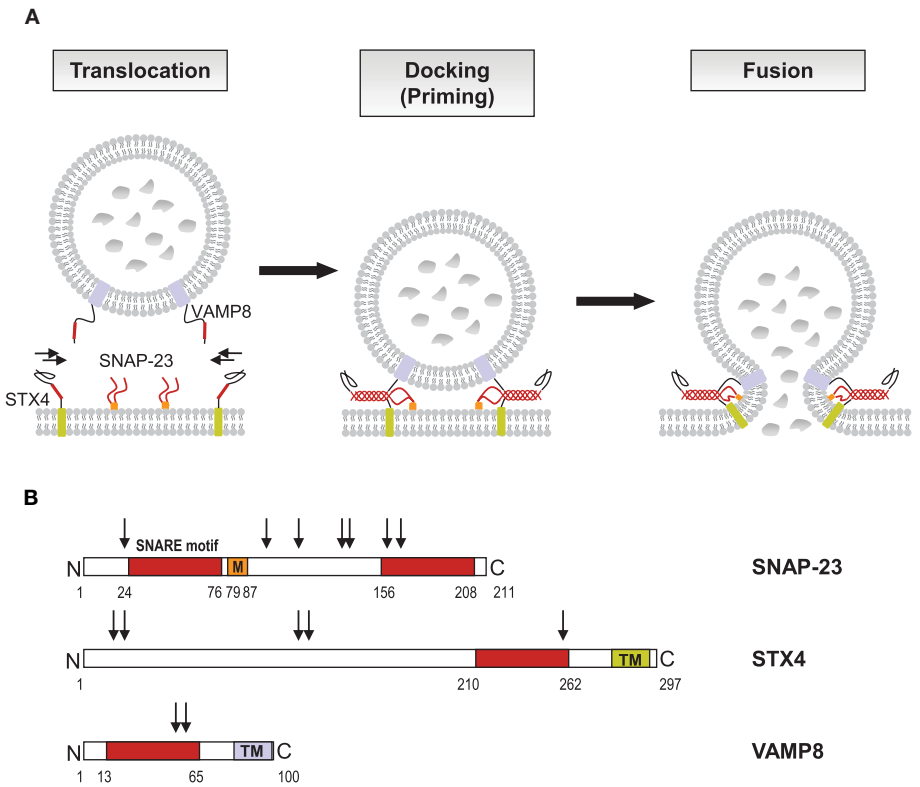

FIGURE 2 | SNARE catalyzed granule fusion in mast cells. **(A)** Secretion of mediators requires fusion of vesicle and plasma membranes. Upon activation through FcεRI secretory granules translocate to and dock at the plasma membrane where the t-SNAREs SNAP-23 and STX4 together with the v-SNARE VAMP8 form stable tetrameric complexes of bundled helices bringing the lipid bilayers into a close distance to catalyze membrane fusion. The SNARE motifs of SNAP-23, STX4, and VAMP8, which become highly organized in the four helical bundle during the formation of the trans-SNARE complex are highlighted in color. **(B)** The primary structure of human SNAP-23, STX4, and VAMP8 as adapted from Hong (2005) is shown with SNARE motifs for each protein in like colors. STX4 and VAMP8 have C-terminal transmembrane domains (TM), whereas the linker domain of SNAP-23, which connects the two SNARE motifs, has a membrane anchor domain, consisting of palmitoylated cysteine residues (M). Numbers indicate protein or domain boundaries, arrows indicate potential phosphorylation sites (http://www.phosphosite.org). Phosphorylation of mouse SNAP-23 on Ser^{95} and Ser^{120} was found to modulate regulated mast cell exocytosis (Hepp et al., 2005), whereas phosphorylation of STX4 was not altered during secretion in RBL cells (Pombo et al., 2001).

was recruited to the raft domain during stimulation, where it was able to form enhanced complexes with SNAP-23 (Puri and Roche, 2006). Furthermore, siRNA-mediated knock-down inhibited IgE-mediated degranulation response (Woska and Gillespie, 2011). Similarly, overexpression of STX4 but not STX2 or STX3 inhibited exocytosis (Paumet et al., 2000). Concerning VAMP proteins several recent studies reported a role of VAMP8, a v-SNARE initially named endobrevin (Wong et al., 1998) due to its localization and function in endosomes and endosomal fusion. The latter underlines the close connection between the endocytic and secretory compartments in mast cells. One study (Tiwari et al., 2008) showed that bone marrow-derived mast cells (BMMCs) derived from VAMP8-deficient mice had reduced release of histamine and β-hexosaminidase while secretion of TNF, CCL2, IL-6, and IL-4 was intact suggesting that VAMP8 acts in pre-stored mediator secretion. The role of VAMP8 was confirmed *in vivo*, as passive systemic anaphylaxis responses were diminished in VAMP8-deficient animals (Tiwari et al., 2008). Similarly, introduction of a soluble recombinant VAMP8 protein into RBL-2H3 cells markedly inhibited release of β-hexosaminidase (Lippert et al., 2007). More recently, studies analyzing the secretion from pancreatic acinar cells suggested that VAMP8 may mediate in particular granule-granule fusion as in these cells only secondary, but not primary fusion events were inhibited in *vamp8* knock-out cells (Behrendorff et al., 2011). While this was not particularly studied in mast cells, it was observed that for VAMP8 both colocalization with granule and with plasma membrane markers increased suggesting that VAMP8 may also participate in both types of fusion events (Tiwari et al., 2008). Another study using VAMP8-deficient BMMC found that VAMP8 controls release of serotonin, cathepsin-D, and β-hexosaminidase, but not of histamine, suggesting that the latter may localize to distinct SGs (Puri and Roche, 2008) rejoining older studies that had reported such differential secretion after depletion of the calcium sensor synaptotagmin II (Baram et al., 1999). On the other hand, both histamine and β-hexosaminidase release were inhibited in BMMC derived from synaptotagmin II-deficient mice (Melicoff et al., 2009). Furthermore, VAMP8 colocalizes with classical SG markers, serotonin,

and mMCP-6 (tryptase; Tiwari et al., 2008). Concerning human mast cells the response profile was largely overlapping with the situation in rodents although some subtle differences became apparent. Indeed, in human mast cells SNAP-23 and STX4 were shown to represent fusion proteins involved in pre-stored mediator release. This was shown after introduction of blocking antibodies into human intestinal-derived mast cells that had been permeabilized with streptolysin O (Sander et al., 2008). Interestingly, in addition to VAMP8, VAMP7 also seemed to play a role as both anti-VAMP7 and anti-VAMP8 were able to reduce histamine release. This rejoins previous data obtained in neutrophils and eosinophils, where secretion of pre-stored mediators examined was inhibited with anti-VAMP7, but not with anti-VAMP8. Secretion in these cells also involved the t-SNAREs STX4 and SNAP-23. In rodent mast cells contradictory studies have been reported with regard to the implication of VAMP7. While one study did not see consistent inhibition after introduction of soluble recombinant VAMP7 in contrast to VAMP8, another study using siRNAs found that both VAMP7 and VAMP8 siRNAs were able to inhibit IgE-mediated degranulation responses in RBL cells. However, no functional studies using VAMP7-deficient mice (Danglot et al., 2012) have been reported so far. Similarly, little information is available for the mechanisms involved in granule-granule fusion. While initial studies by Guo et al. suggested a relocation mechanism of the plasma membrane SNARE into nascent degranulation channels, it is possible that granule-granule fusion may be mediated by different types of SNARE protein complexes. They may include for example STX3, which has been reported to localize in rodent cells both to SG and the plasma membrane (Hibi et al., 2000; Martin-Verdeaux et al., 2003). An implication of STX3 is also suggested by the role Munc18-2 plays in granule exocytosis (see below) as the latter specifically binds to STX3, but not to STX4 (Martin-Verdeaux et al., 2003).

SNARE PROTEINS IN CYTOKINE/CHEMOKINE SECRETION

The role of SNARE proteins in the release of *de novo* synthesized mediators like cytokines or chemokines from mast cells has hardly been analyzed. However, our knowledge is also accumulating thanks to studies performed in other immune cells. For a generalized review the reader is referred to some excellent recent reviews (Huse et al., 2008; Lacy and Stow, 2011). The data so far indicate that chemokine/cytokine secretion does not follow a unique pathway but rather represents a complex machinery of multiple pathways and organelles. In T cells both a polarized directional pathway to the synapse (whisper) involving the t-SNAREs SNAP-23 and STX4 as well as multi-directional pathways (shout) involving STX6 containing vesicles have been described (Huse et al., 2006). The vesicular carriers of the former are tightly coupled to the accompanying microtubular reorganization, while for the latter fusion occurs at multiple sites at the plasma membrane as shown for life imaging of TNF secreting cells in the presence of tumor necrosis factor-α-converting enzyme (TACE) inhibitors that prevent the cleavage of the membrane precursors by the metalloproteinase TACE (also called ADAM17) once they have reached the plasma membrane. Many immune cells are also able to store chemokines/cytokines in cytoplasmic storage organelles from where they get mobilized. The first description of granule-pre-stored cytokines stems from Gordon and Galli (1990) in mast cells. They noticed that certain types of mast cells contain high amounts of pre-stored TNF that get rapidly released upon stimulation. The cytoplasmic storage organelles may correspond to classical mediator-containing SG allowing the rapid release together with other inflammatory mediators (Pelletier et al., 1998). Since then many other chemokines/cytokines have been reported to be pre-stored in cytoplasmic vesicles including besides mast cells for example neutrophils and eosinophils (Bjerke et al., 1996; Calafat et al., 1997; Mahmudi-Azer et al., 2000). In eosinophils the release of CCL5 and IL-4 may involve the mechanism of piecemeal degranulation whereby CCL5/IL-4 present in crystalloid granules get mobilized by forming small vesicular carriers transported to the plasma membrane (Lacy and Stow, 2011).

Another major pathway could be the constitutive release that is fed by the increased transcription and translation of chemokines/cytokines in response to a stimulatory event. The prototype mechanism may be the secretion of newly synthesized TNF by macrophages, which is reported to be organized in two steps (Stow et al., 2006, 2009; Lacy and Stow, 2011). First, the fusion of carriers originating from the Golgi complex with recycling endosomes is mediated by the Q-SNARE complex of STX6-STX7-Vti1b with the R-SNARE VAMP3, and second, the membrane fusion of the recycling endosome and the plasma membrane is mediated by the R-SNARE VAMP3 on the recycling endosome by pairing with the STX4–SNAP-23 Q-SNARE complex on the plasma membrane (Pagan et al., 2003; Murray et al., 2005a,b). On the other hand early studies performed in mast cells examining TNF secretion clearly showed the necessity for a stimulatory calcium signal or activation of PKC as addition of selective pharmacological blockers after some time completely arrested secretion despite the fact that TNF had already accumulated within the cell (Baumgartner et al., 1994).

VAMP8 was the first SNARE protein examined for a role in cytokine/chemokine trafficking in mast cells. As already mentioned above, we and others found in BMMC that absence of VAMP8 did not affect secretion of several chemokines or cytokines tested contrasting with some recent data showing an implication of VAMP8 in anaphylatoxin-induced TNF release in macrophages (Tiwari et al., 2008; Pushparaj et al., 2009). For the latter its implication in cytokine release was specific for TNF as IL-1β, IL-6, and CCL3 were not affected. In wild-type macrophages, TNF was found to colocalize with VAMP8-positive vesicles, and in VAMP8-deficient macrophages, TNF release was inhibited (Tiwari et al., 2008; Pushparaj et al., 2009). Furthermore, VAMP8 has been shown to regulate the release of TNF and β-hexosaminidase in macrophages triggered by fMLP (Alvarez de Toledo and Fernandez, 1990). In mast cells, TNF did not colocalize with VAMP8-containing vesicles, but was rather found to colocalize with a VAMP3 positive compartment in a manner similar to the compartments described for release of TNF into the phagocytotic cup (Tiwari et al., 2008). Given that VAMP3 has been associated with the recycling endosomal compartment this opens the possibility of trafficking through such a compartment prior to release. Interestingly, further studies in human mast cell lines showed that TNF traffics to the membrane, from where it gets re-endocytosed into cytoplasmic granules suggesting that granular localization could

depend on a specific mechanism of re-endocytosis (Olszewski et al., 2007) although direct sorting *via* a Mannose phosphate receptor-dependent pathway has also been proposed in rodent mast cells (Olszewski et al., 2006). On the other hand, when analyzing BMMC, we did not see significant granule localization by probing with an antibody detecting endogenously produced TNF and rather found TNF co-localized with VAMP3-containing fractions. Yet, in some isolated cells we were also able to detect TNF in SG. This agrees with previous data showing that in BMMC a small, but detectable fraction (<10%) of TNF gets mobilized rapidly being in line with a SG storage mode (Gordon and Galli, 1991). **Figure 3** summarizes possible cytokine secretion pathways in mast cells.

More recently we examined the implication of SNARE proteins in chemokines secretion from human intestinal-derived mast cells reported to represent a potent source of many different human chemokines (Feuser et al., 2012). We found that blocking antibodies directed to SNAP-23 and STX3, but not STX2 and VAMP3, inhibited release from all chemokines tested (CXCL8, CCL2, CCL3, and CCL4; Frank et al., 2011). It should be noted that in human mast cells STX3, like STX4, is localized at the plasma membrane, while in rodent mast cells it is found mainly on SG. By contrast a differential behavior was noted concerning other SNARE proteins tested. STX4 and VAMP8 were found to play a specific role in CCL8 secretion while anti-STX6 selectively inhibited CXCL8 and CCL2 and anti-VAMP7 CCL3. Thus, similar to the findings observed in macrophages, the release of *de novo* synthesized cytokines by mast cells seems to involve more than three or four SNARE proteins. Although the situation concerning exocytosis is in principle different in mast cells and macrophages, the release of cytokines could be similarly organized. In human mast cells chemokine release could be mediated by a Q-SNARE complex consisting of SNAP-23, STX3, or STX4 and the R-SNARE VAMP7 or VAMP8 at the plasma membrane. A second composition could involve a Q-SNARE complex consisting of Vti1b and STX6 together with STX3 or STX4. However, we found that VAMP8 is involved at least in the release of CXCL8 by human mast cells, whereas VAMP3 surprisingly was not involved in any release (Sander et al., 2008; Frank et al., 2011). In summary, our data suggest that vesicular carriers involved in chemokine secretion show a highly heterogenous profile that needs to be further characterized.

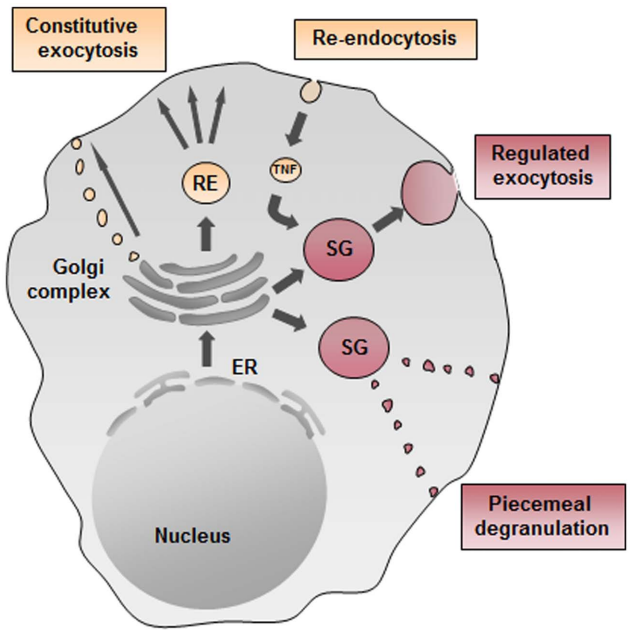

FIGURE 3 | Possible cytokine secretion pathways in mast cells. Cytokine secretion may occur constitutively through small vesicular carriers or through recycling endosomes (ER) as described for TNF in macrophages. Moreover, TNF could get re-endocytosed from the plasma membrane and transported into secretory granules (SG) and then rapidly released upon stimulation. Another possible pathway of regulated exocytosis in mast cells may be piecemeal degranulation as reported for eosinophils.

MUNC18 PROTEINS IN THE REGULATION OF EXOCYTOSIS

In contrast to SNARE proteins, less information is available concerning the function of Sec1/Munc18 (SM) proteins known to play fundamental roles in various intracellular secretory trafficking steps (Sudhof and Rothman, 2009). Sec1 mutant yeast cells have been initially identified in a genetic screen in yeast aiming to identify proteins involved in the secretory pathway (Novick et al., 1980). A defect in neurotransmitter release was also apparent for the *uncoordinated18* (unc18) ortholog in nematodes (Hosono et al., 1992) and the drosophila Rop protein (Harrison et al., 1994). SM proteins contain seven family members in humans that bind to STX SNAREs (Bock et al., 2001). Three family members are *mammalian uncoordinated18* (Munc18) proteins that are more specifically implicated in regulated exocytosis. They include Munc18-1 (sometimes also called Munc18a; **Figure 4A**), which is largely expressed in neurons and two ubiquitously expressed isoforms Munc18-2 (Munc18b) and Munc18-3 (Munc18c). They show specificity of binding for certain STX family members. Thus, Munc18-1 can interact with STX1, 2, and 3, Munc18-2 with STX1, 3, and slightly with STX2 (Hata and Südhof, 1995); Munc18-3 interacts with STX2 and 4 and to a lesser extent with STX1 (Tellam et al., 1995). Recently, Munc18-2 was also shown to interact with STX11 (Cote et al., 2009). Studies in knock-out mice have clearly underlined the important role of Munc18 proteins in regulated exocytosis. Animals deficient in Munc18-1 have a complete block of neurotransmission and mice die at birth due to a breathing defect (Verhage et al., 2000). Mice deficient in Munc18-3 are early embryonic lethal and in the heterozygous stage are glucose intolerant after receiving high-fat-diet due to a secretion defect in insulin-producing pancreatic beta cells (Oh et al., 2005). On the other hand, externalization of GLUT4 at the cell surface was enhanced in *munc18-3* knock-out adipocytes at low concentration of insulin (Kanda et al., 2005). The reported essential role of Munc18 proteins in the secretory pathway was initially difficult to explain as biochemical experiments as well as crystal structure analysis of the STX1/Munc18-1 complex showed that Munc18 binding to their respective STX partners blocked the interaction with other SNAREs rather arguing for a negative regulatory function (Araki et al., 1997; Misura et al., 2000). This problem was solved by the discovery that Munc18-1 in addition of binding to the closed conformation of STX1 was also able to bind to the assembled tetrameric SNARE complex (Sudhof and Rothman, 2009).

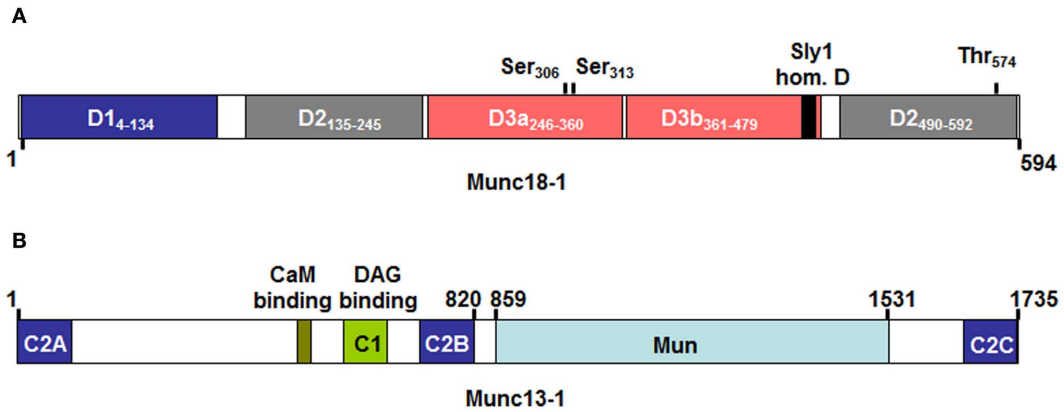

FIGURE 4 | Domain structure of Munc18-1 (A) and Munc13-1 (B) as adapted from Koch et al. (2000); Misura et al. (2000); Li et al. (2011). Numbers above indicate protein or domain boundaries. For Munc18-1 domain boundaries are also directly included in the inset. The Munc18-1 protein contains three domains (with domain 3 being divided in D3a and D3b). According to crystal structure analysis the molecule adopts a horseshoe like structure that holds the STX-bound molecule in its closed conformation. Contact surfaces reside in D1 and D3a, which form the bottom ends of the horseshoe, while D2 and D3b form the upper end. Domain 3b includes the so-called Sly 1 homology domain containing the residue homologous to the Sly1-20 mutant in the yeast Sly1 protein, which bypasses the requirement for a Rab effector protein in yeast vesicular transport (Dascher et al., 1991). Above the domains are also indicated protein kinase C (Ser^{306} and Ser^{313}) and Cyclin dependent kinase (Thr^{574}) phosphorylation sites that may regulate Munc18-1 effector functions. **(B)** The Munc13-1 protein contains several calcium-binding C2 domains, a Diacylglycerol (DAG) C1 domain making it responsive to stimulation with PKC as well as a calmodulin binding domain. Note that the ubiquitouisly expressed isoform 13-4, which is important in mast cell exocytosis lacks the N-terminal C2a, CaMb, and C1 domains. The Mun domain shows structural similarity to thethering proteins and may play a role in the transition of the closed conformation of STX molecules by contacting either Munc18 or STX molecules (Li et al., 2011; Sudhof and Rizo, 2011).

It was proposed that Munc18-1 switches its binding mode during the Munc13-regulated (see below) conformational transition of Syntaxin proteins to their "open" form that allows engagement with other SNARE partners (Sudhof and Rizo, 2011). Once the transition completed Munc18 directly participates in the fusion reaction. Phosphorylation events mediated by protein kinase C shown to phosphorylate Ser^{316} and Ser^{313} in Munc18-1 as well as cyclin dependent kinase phosphorylating Thr^{574} (**Figure 4A**) may also participate in such regulatory events (Fletcher et al., 1999; Snyder et al., 2006; Wierda et al., 2007). The fusion enhancing role of Munc18 was directly demonstrated in biochemical reconstitution experiments (Shen et al., 2007). Furthermore, structural data showed that Munc18-1 binds the assembled SNARE complex at the C-terminal end close to membranes suggesting that Munc18 might cooperate with the SNARE complex by providing a supplementary pulling force (Carr and Rizo, 2010). Nevertheless, its physiological function may even be more complex as Munc18 proteins may promote fusion also by other mechanism that may relate to their ability to interact with other fusion regulatory proteins such as Mints (Ho et al., 2006), Doc2 (Higashio et al., 2008), Granuphilin (Fukuda et al., 2005), or Cab45 (Lam et al., 2007). Besides promotion of fusion it has been reported that Munc18 proteins also regulate steps such as vesicle translocation, vesicle tethering and vesicle docking (Burgoyne et al., 2009).

Mast cells were found to express the ubiquitous isoforms Munc18-2 and Munc18-3 (Martin-Verdeaux et al., 2003). The neuronal isoform Munc18-1 has also been detected, however, so far only at the mRNA level (Nigam et al., 2005). In co-immunoprecipitation experiments Munc18-2 interacted with STX3 and less so with STX2, while Munc18-3 interacted with STX4 (Martin-Verdeaux et al., 2003). Interestingly, in contrast to the neuronal isoform, Munc18-2 was found to be localized to SG, which is, however, in agreement with the granular localization of its STX3 binding partner (**Figure 5**). Munc18-3 was localized at the plasma membrane in agreement with the plasma membrane localization of its binding partner STX4. Functional studies have so far revealed only a role for Munc18-2. Expression of peptides corresponding to an activation loop or overexpression of wild-type proteins in RBL cells was able to inhibit regulated exocytosis (Martin-Verdeaux et al., 2003). By contrast, no biological function was shown after expression of corresponding peptides or proteins of Munc18-3. Recent studies using a siRNA-mediated knock-down of Munc18-2 also blocked inflammatory mediator secretion in RBL mast cells (Tadokoro et al., 2007). The mechanism of action has not been investigated. Yet it was interesting to note that in the presence of microtubule-depolymerizing agents one could observe a redistribution of Munc18-2 from secretory vesicles to the cytoplasm, indicating a connection with the microtubule cytoskeleton (Martin-Verdeaux et al., 2003). In that respect, neuronal Munc18-1 co-purified and co-localized with cytoskeletal proteins such as α and β tubulin (Bhaskar et al., 2004). New data also show a coupling of STX1/Munc18-1 to the microtubule motor protein Kinesin *via* the FEZ1 adaptor protein further confirming the tight connection of Munc18-1 with this cytoskeletal compartment (Chua et al., 2012). Munc18 isoforms have also been shown to function in other immune cells. In neutrophils, Munc18-2 could act as a regulator of primary granule exocytosis, while Munc18-3 may preferentially regulate the fusion of secondary/tertiary granules. Recently, human familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) patients were shown to contain mutations within Munc18-2 that led to a deficiency in expression of Munc18-2 and concomitantly STX11 (Cote et al., 2009; Zur Stadt et al.,

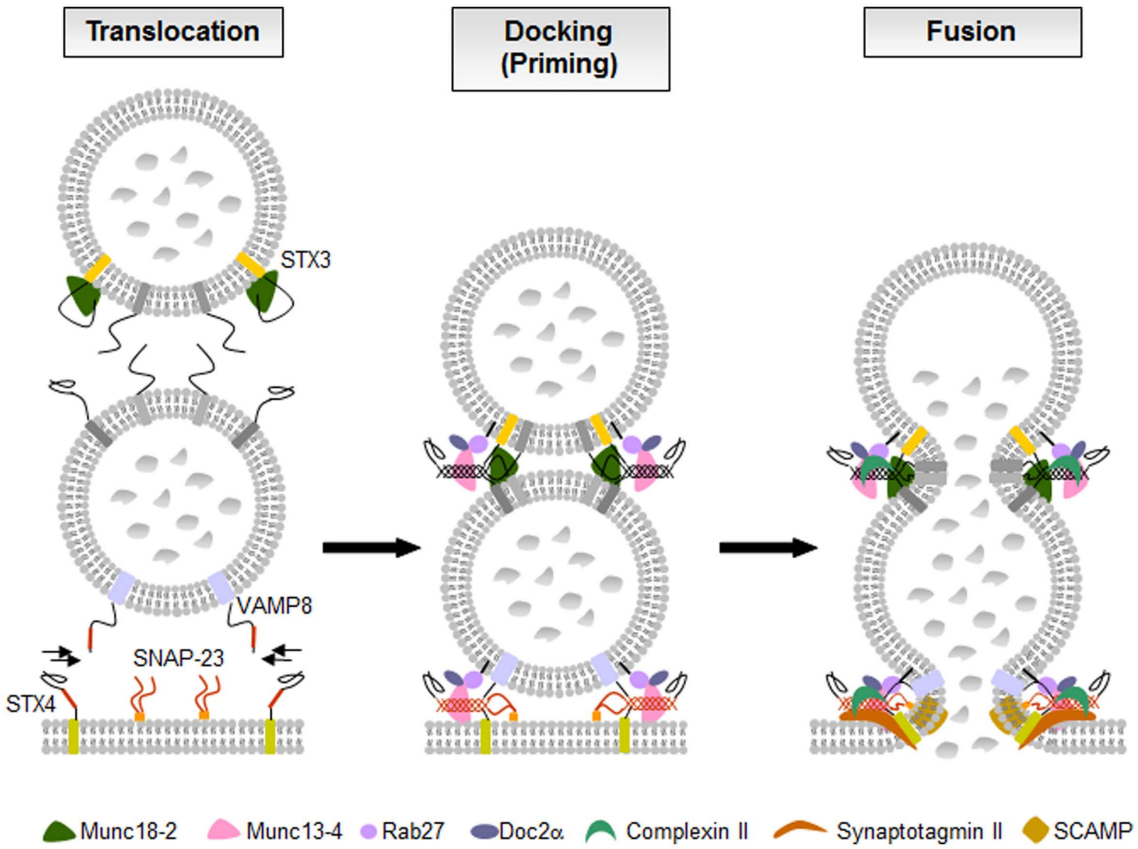

FIGURE 5 | The fusion machinery in mast cell secretion. In resting cells membrane fusion is blocked likely by molecules such as STX3-bound Munc18-2. Upon activation through FcεRI secretory granules translocate to and dock at the plasma membrane where the SNARE proteins assemble into tetrameric complexes. Multiple accessory proteins such as Munc18-2, Munc13-4, Rab27, Doc2α, complexin II, synaptotagmin II, or SCAMPs, are involved as "gatekeepers" of the fusion reaction holding effectors in an inactive state, as tethering factors controlling docking and priming, or as regulators of the SNARE complexes triggering the calcium-dependent membrane fusion.

2009). Like in FHL4 patients, who have mutations in STX11 (Zur Stadt et al., 2005), this caused lytic granule exocytosis in NK and cytotoxic T cells, albeit the phenotype was largely dependent on the type of mutation.

MUNC13 PROTEINS IN REGULATED EXOCYTOSIS

In addition to SNARE and SM proteins, Munc13 protein family members have also been demonstrated to play an essential role in vesicle fusion where they are thought to be involved in vesicle priming. Munc13 are large active zone proteins containing multiple domains (**Figure 4B**) including phospholipids and calcium-binding C2 domains, a phorbol ester binding C1 domain, and a large Mun domain homologous with tethering factors (Koch et al., 2000; Li et al., 2011; Sudhof and Rizo, 2011). Genetic ablation of neuronal isoforms (Munc13-1, -2, and -3) in mice or the *C. elegans* homolog unc-13 led to severe defects in both spontaneous and evoked synaptic transmission due to a defect in vesicle priming (Augustin et al., 2001; Rosenmund et al., 2002). Similarly, mutations in the ubiquitously expressed Munc13-4 isoform showed that granules in cytotoxic T cells although appear to dock do not fuse leading to type 3 familial hemophagocytic lymphohistiocytosis (FLH3; Feldmann et al., 2003). The fact that priming defects could be rescued with a STX1 mutant promoting a constitutively "open" form of STX1 (Richmond et al., 2001) suggested that Munc13 isoforms are involved in the transition of the closed form of STX to its open form that also allows Munc18-1 to adapt its transition to the new binding mode able to bind the assembled trans-SNARE complex. This function was confirmed using NMR and fluorescence spectroscopic as well as crystal structural studies (Li et al., 2011; Ma et al., 2011). Recruitment of Munc13 to the membrane and fusion machinery may be mediated by its capacity to interact with Rab proteins such as Rab3 for neuronal-expressed Munc13 isoforms or Rab27a in cytotoxic T cells (Menager et al., 2007). Indeed, recent studies identified in Munc13-4 a region at the C-terminal end of its first C2A domain involved in Rab27a binding (Elstak et al., 2011). This domain was required in order for Munc13-4 to support degranulation notably by restricting the motility and by securing the tight docking of SG at the plasma membrane. Studies in cytotoxic T cells showed that in addition to its role as a priming factor Munc13-4 also promotes the fusion of late endosomes with recycling endosomes to form secretion-competent secretory vesicles indicating additional roles for this protein (Menager et al., 2007).

Concerning mast cells, several studies indicated that like in cytotoxic T cells Munc13-4 has important functions. Indeed, both Munc13-4 and Rab27 isoforms Rab27A and Rab27B are highly expressed in mast cells and both proteins were shown to interact (Neeft et al., 2005). Overexpression of Munc13-4 enhanced degranulation indicating a positive regulatory function. In agreement siRNA-mediated knock-down inhibited degranulation and transfection with wild-type, but not Rab27-deficient binding mutants could rescue the inhibitory phenotype (Elstak et al., 2011). This indicates that like in cytotoxic T cells Munc13-4 could promote fusion by stabilizing docked vesicles at the plasma membrane. In addition to Rab27 isoforms, Munc13-4 was also shown to interact with the calcium and phospholipids-binding C2 domain containing effector Doc2α in mast cells (Higashio et al., 2008). Doc2α interacts with Munc13-4 *via* two distinct N- and C-terminal located regions (Higashio et al., 2008). The importance of Doc2α is underlined by the marked inhibition of exocytosis in Doc2α-deficient BMMC (Higashio et al., 2008). Furthermore, expression of mutants lacking the Munc13-4 interacting domains impaired secretion after transfection. These mutants were also unable to rescue secretion after siRNA-mediated knock-down of Doc2α in contrast to wild-type Doc2α. Taken together, these results support an important role of Munc13-4 in mast cell degranulation. Munc13-4 likely acts together with other fusion accessory proteins such as Rab27 isoforms and Doc2α to stabilize vesicle docking and prime them for fusion (**Figure 5**). The latter could in part be dependent on its ability to catalyze the transition of the STX/Munc18 complex to an "open" conformation ready to engage with other SNARE partners.

ROLE OF OTHER ACCESSORY PROTEINS IN THE REGULATION OF EXOCYTOSIS

Since it has been realized that there must be other factors for a complete and fast vesicle fusion apart from SNARE proteins, a long list of other accessory regulators such as Rab GTPases, complexins, synaptotagmins, or secretory carrier membrane proteins (SCAMPs) were found in addition to SM and Munc13 proteins.

Rab GTPases are low-molecular-weight GTPases, which function as molecular switches that alternate between two conformational states: the GTP-bound "on" form and the GDP-bound "off" form (Stenmark, 2009). Rab GTPases are reversibly associated with membranes and involved in regulating membrane traffic. More than 70 Rab proteins have been identified in mammalian cells up to now (Hutagalung and Novick, 2011). Mast cells express different members of the Rab3 and Rab27 family. RBL-2H3 mast cells were found to express Rab3 isoforms with Rab3D being the most prevalent (Roa et al., 1997; Tuvim et al., 1999). Moreover, Rab3D was found to localize on mast cell SG and to translocate to the plasma membrane upon exocytosis suggesting that Rab3D is a component of the regulated exocytosis in mast cells (Tuvim et al., 1999). Maybe, it is involved in controlling SNARE assembly in a calcium-dependent manner (Pombo et al., 2001). However, mast cell secretion in *rab3d* knock-out mice was unaffected, although compensatory mechanisms by other isoforms have not been investigated (Riedel et al., 2002).

Murine BMMCs express both Rab27a and Rab27b isoforms, which localize to SG (Mizuno et al., 2007; **Figure 5**). Rab27a and its effector Munc13-4 were described to be involved in exocytosis in RBL-2H3 cells (Goishi et al., 2004; Neeft et al., 2005). The Munc13-4-Rab27a complex has been identified to be required for tethering of secretory lysosomes to the plasma membrane and controlling their docking that is necessary for degranulation in immune cells (Elstak et al., 2011). However, secretory defects were only present in *rab27b* knock-out and *rab27a/rab27b* double knock-out mice, but not in *rab27a* knock-out mice (Mizuno et al., 2007). Immunofluorescence studies indicate that a subset of Rab27b-deficient BMMCs exhibit mild clustering of granules. Double Rab27-deficient BMMCs showed almost 10-fold increase of granules in microtubule-dependent movement suggesting that Rab27 proteins, particularly Rab27b, regulate the transition from microtubule to actin-based motility and are crucial for mast cell degranulation (Mizuno et al., 2007).

Complexin I and II are small soluble cytosolic proteins and interact with SNARE complexes (Sudhof and Rothman, 2009). Knock-out mice showed reduced neurotransmitter release suggesting that complexin acts as a positive regulator of exocytosis (Reim et al., 2001). On the other side, injection or overexpression of complexin reduced neurotransmitter or exocytotic release and injection of anti-complexin antibodies stimulated neurotransmitter release suggesting that complexin is a negative regulator (Ono et al., 1998; Itakura et al., 1999). In neuronal synapses, complexin was found to simultaneously suppress spontaneous fusion and activate fast calcium-evoked fusion. The dual-function was dependent on SNARE binding and N-terminal sequences of complexin that localize to the point where SNARE complexes insert into the fusing membranes, suggesting that complexin controls the force that SNARE complexes apply onto the fusing membranes (Maximov et al., 2009).

RBL-2H3 cells express complexin II, but not complexin I (Tadokoro et al., 2005). Complexin II knock-down experiments revealed that complexin II positively regulated exocytotic release in mast cells by translocating to the plasma membrane and enhancing the calcium sensitivity of the fusion machinery (Tadokoro et al., 2005). The association of complexin II with SNARE complex (**Figure 5**) was not sufficient to trigger exocytotic membrane fusion. *In vitro* binding assays showed that complexin II interacts with SNARE complex containing STX3 to regulate mast cell degranulation, but does not bind to SNARE complex containing STX4 (Tadokoro et al., 2005). STX3 is found on the plasma membrane as well as on SG and thus might be involved in both the fusion with the plasma membrane and granule–granule fusion to facilitate exocytotic release in mast cells (Hibi et al., 2000; Martin-Verdeaux et al., 2003; Tadokoro et al., 2007, 2010). The calcium sensor synaptotagmin is thought to finally trigger membrane fusion by reversing the complexin block on activated SNARE complexes in addition to its calcium-dependent phospholipid-binding activity (Maximov et al., 2009).

Synaptotagmins are calcium-binding proteins containing two conserved binding domains at their C-terminus, C2A and C2B, and a single N-terminal transmembrane domain anchored to membranes of secretory vesicles (Sudhof, 2004). The binding of calcium to the C2 domains alters their electrostatic surface charge and mediates most of the calcium-dependent functions of synaptotagmin (Chapman, 2008). It is thought that synaptotagmin

triggers fusion by binding to SNARE complexes on the target membrane with its C2A domain and undergoes calcium-dependent self-oligomerization of its C2B domain, accompanied by interaction with negatively charged membrane phospholipids. SNAREs are able to form complexes in the absence of calcium, but the arrangement by synaptotagmin in response to calcium is essential for the fusion event (Chapman, 2008).

Several isoforms of synaptotagmin such as synaptotagmin II, III, V, or IX are expressed in mast cells (Baram et al., 2001; Haberman et al., 2007). Synaptotagmin II was identified as the major isoform expressed in RBL-2H3 cells (Baram et al., 1999) and found to regulate exocytosis but not other secretory responses of mast cells (Melicoff et al., 2009; **Figure 5**). Synaptotagmin II-deficient mice had normal mast cells in number, morphology, and structure or storage of granule contents. BMMCs generated from these mutant mice had no defects in IgE-dependent generation and secretion of cytokines and eicosanoids, but had a marked deficiency in the exocytosis of their pre-formed granule mediators (Melicoff et al., 2009). Using a liposome-based fusion assay it was shown that synaptotagmin II regulates membrane fusion of SNARE-containing liposomes, and that this regulation is dependent on synaptotagmin II concentration, Ca^{2+}, and phosphatidylserine (Nagai et al., 2011). Synaptotagmin III has been found to be required for the formation of the endocytic recycling compartment (ERC), which is involved in sorting of proteins from SG and connecting the endocytic pathway with the exocytic one (Sagi-Eisenberg, 2007). Synaptotagmin IX was found to be endogenously expressed and ERC-localized in mast cells (Haberman et al., 2003, 2005). Knock-down of synaptotagmin IX was associated with mistargeting of TGN38, a protein that normally cycles between the trans-Golgi network, the ERC, and the plasma membrane, and its delivery to the SG. Thus, synaptotagmin IX is a part of the machinery that is involved in the formation of transport carriers that mediate SG protein sorting (Haberman et al., 2007).

Secretory carrier membrane proteins are conserved four transmembrane-spanning proteins associated with recycling vesicular carriers. Five distinct SCAMPs have been reported in mammals with SCAMPs 1–3 being expressed in mast cells (Castle et al., 2002). SCAMP1 and 2, the most prevalent SCAMPs in mast cells, are present in SG membranes and other intracellular membranes. A small population was found to partially colocalize with SNAP-23 and STX4 (Guo et al., 2002). Administration of the E peptide, an oligopeptide within the cytoplasmic segment linking the second and third transmembrane spans, particularly of SCAMP2, potently inhibits exocytosis in streptolysin O-permeabilized mast cells. It blocks fusion beyond the docking step where granules contact the cell surface and each other during compound exocytosis suggesting that SCAMP2 may play a critical role in completing exocytosis (Guo et al., 2002; **Figure 5**). SCAMPs may also act at a step to form fusion pores because in neuroendocrine PC12 cells SCAMP2 interacts with Arf6 and Phospholipase D1 coupling Arf6-stimulated PLD activity to the exocytotic fusion pore formation (Liu et al., 2002). **Table 1** summarizes SNAREs and accessory proteins found in mast cells.

THE FUSION MACHINERY AND CELL SIGNALING

Many, if not all proteins of the membrane fusion machinery contain sequences amenable to post-translational modifications such as phosphorylation, lipid modifications, and nitrosylation. These events have been shown to be involved in the regulation of SNARE complex formation connecting it to cell signaling (Matsushita et al., 2003; Snyder et al., 2006; Davletov et al., 2007). One important example in mast cells is the phosphorylation of the t-SNARE protein SNAP-23 that had been shown to be critically involved in the regulation of fusion. Indeed, a sizable fraction (10%) of SNAP-23 becomes transiently phosphorylated during degranulation on Ser^{95}/Ser^{120} within its cysteine-rich linker region (Hepp et al., 2005; **Figure 2B**). Interestingly, this involved Ikappa B kinase 2 (IKK2), an enzyme previously known to regulate nuclear translocation of the NF-κB transcription factor through the phosphorylating cytoplasmic inhibitor IkB (Suzuki and Verma, 2008). Indeed, IKK2-deficient mast cells showed a strong impairment of their capacity to degranulate and mediate anaphylactic responses *in vivo*. This was independent of their capacity to activate NF-κB and could be rescued in the presence of a phosphomimetic mutant of SNAP-23, while wild-type SNAP-23 was ineffective. Furthermore, in agreement with the demonstration that phosphorylated SNAP-23 was present in enhanced amounts in SNARE complexes, IKK2 was recruited to lipid raft domains, which are the sites of membrane fusion in mast cells. Secretion by mast cells is also regulated by PKC. In particular, BMMC deficient in the calcium-dependent isoform PKCβ are strongly impaired in their capacity to degranulate (Nechushtan et al., 2000). However, the precise molecular targets remain unknown. In addition to kinases, phosphatases were also shown to regulate components of the fusion machinery. Thus, megakaryocyte cytosolic protein tyrosine phosphatase 2 (MEG2) has been described to dephosphorylate NSF on a key tyrosine residue (Huynh et al., 2004). In RBL mast cells MEG2 localized to SG and its overexpression resulted in the formation of large granules (Wang et al., 2002).

Many of the proteins of the fusion machinery also contain domains responding to calcium, one of the most widely used second messengers in cell signaling. Prominent calcium sensors of exocytosis are the synaptotagmin family of proteins. Upon calcium binding to its two tandem C2 domains, neuronal-expressed synaptotagmin I has been shown to undergo an electrostatic switch that promotes oligomerization and phospholipid-binding. This promotes the interaction with membrane lipids causing membrane bending, which facilitates fusion (Chapman, 2008). A similar action was proposed for Doc2α, which also contains two tandem C2 domains. Due to its different calcium binding characteristics and affinity the latter was involved in the asynchronous release, which is less tightly coupled to the action potential (Yao et al., 2011). Both types of sensors are expressed in mast cells and thus likely couple vesicle release to calcium signaling, albeit the mechanisms and connections between these effectors, calcium regulation and the SNARE fusion machinery needs to be studied in detail. Another calcium-regulated activity is Rak3D, a kinase associated with the small GTPase Rab3D (Pombo et al., 2001; Coppola et al., 2002). *In vitro*, Rak3D was able to phosphorylate STX4, but not STX2 and STX3, thereby decreasing its capacity to interact with

Table 1 | Fusion and fusion accessory proteins in mast cells.

Group	Name	mRNA	Protein expression	KO model; human deficiency	Subcellular location in mast cells	Functional evidence for a role in mast cell exocytosis	Reference
t-SNAREs	SNAP-23	+	+	KO + (lethal)	PM	SNAP family representative expressed in murine and human MC. Required for release of pre-stored and cytokine/chemokine products. Forms with VAMP8 and STX4 the major SNARE complex in activated RBL-2H3 cells. SNAP-23 siRNA treatment impairs antigen-induced MC degranulation. SNAP-23 inhibition decreases IgE-mediated histamine release in human MC.	Blank (2011), Frank et al. (2011), Sander et al. (2008), Vaidyanathan et al. (2001), Woska and Gillespie (2011)
	SNAP-25	+	±	KO + (lethal)	SG	Human, murine, rat MC (low levels)	Hodel (1998), Salinas et al. (2004), Sander et al. (2008)
	STX2	+	+		ND	Overexpression has no effect on degranulation. Inhibition has no effect on chemokine release.	Paumet et al. (2000), Frank et al. (2011), Sander et al. (2008), Woska and Gillespie (2011)
	STX3	+	+		SG, PM	Contributes to both SG-PM and SG-SG fusion. Crucial for the release of cytokines/chemokines from mature human MC. Direct binding of complexin II. SNARE complex comprises SNAP-23, STX3, VAMP8, and complexin II.	Frank et al. (2011), Hibi et al. (2000), Paumet et al. (2000), Sander et al. (2008), Tadokoro et al. (2007, 2010), Tiwari et al. (2008)
	STX4	+	+	KO + (lethal)	PM	Forms with VAMP8 and STX4 the major SNARE complex in activated RBL-2H3 cells. Interacts with SNAP-23, VAMP7, or VAMP8 upon activation of human MC. STX4 inhibition results in decreased IgE-mediated histamine release from human MC. STX4 siRNA treatment impairs antigen-induced MC degranulation. STX4 inhibition decreased release of CXCL8 but not CCL2, CCL3, or CCL4.	Frank et al. (2011), Paumet et al. (2000), Sander et al. (2008), Woska and Gillespie (2011)
	STX6	+	+		ND	Demonstrated in human MC.	Sander et al. (2008)
v-SNAREs	VAMP2 (synaptobrevin)	+	+	KO+	SG	Low levels in human MC. Minor colocalization with SG, relocation from SG to PM in activated MC. VAMP2 siRNA does not modulate RBL-2H3 cell degranulation. Compensatory effect through enhanced SNAP-23 – VAMP2 complex formation in activated VAMP8−/− derived MC.	Puri and Roche (2008), Sander et al. (2008), Tiwari et al. (2008), Woska and Gillespie (2011)
	VAMP3	+	+	KO+	Vesicular, SG	Substantial amounts in human MC. Minor colocalization with SG; major relocation to PM in IL-1beta stimulated MC in absence of degranulation Peripheral VAMP3 colocalizes with exocytosed TNF at the cell surface. No effect on chemokine release. No effect on IgE-mediated histamine release.	Frank et al. (2011), Paumet et al. (2000), Puri and Roche (2008), Sander et al. (2008), Tiwari et al. (2008)

(Continued)

Table 1 | Continued

Group	Name	mRNA	Protein expression	KO model; human deficiency	Subcellular location in mast cells	Functional evidence for a role in mast cell exocytosis	Reference
	VAMP7	+	+	KO+	Vesicular	Substantial amounts in human MC. Minor colocalization with SG. Relocation to PM in activated MC. Interacts with SNAP-23 and STX4 in activated MC. VAMP7 inhibition decreases IgE-mediated histamine release from human MC. VAMP7 siRNA treatment impairs IgE-induced MC degranulation.	Hibi et al. (2000), Sander et al. (2008), Tiwari et al. (2008), Woska and Gillespie (2011)
	VAMP8 (endobrevin)	+	+	KO+	Vesicular, SG	Substantial amounts in human MC. Localizes to endosomes, but also major colocalization with SG. Relocation to PM upon MC activation. *VAMP8*−/− MC display impaired degranulation of pre-stored mediators *in vitro* and *in vivo*, but normal cytokine and chemokine production. Forms increased complexes with SNAP-23 and STX4 after stimulation. Specific cytokine/chemokine release by IL-1beta stimulation in the absence of degranulation does not induce VAMP8 relocation to PM.	Frank et al. (2011), Paumet et al. (2000), Hibi et al. (2000), Puri and Roche (2008), Sander et al. (2008), Tadokoro et al. (2010), Tiwari et al. (2008), Woska and Gillespie (2011)
Accessory proteins	Rab3A	+	+		Cytoplasmic	Overexpression: either null or inhibitory effect on activated MC exocytosis.	Roa et al. (1997), Smith et al. (1997)
	Rab3B	+	+		ND	ND	Oberhauser et al. (1992)
	Rab3D	+	+	KO+	SG, others	Relocation to PM upon MC activation. May regulate SNARE assembly *via* Rak3D-mediated phosphorylation of STX4. Rab3D deficiency does not impair exocytosis in patch clamp activated MC. Rab3D deficiency is associated with abnormally large SG, suggesting a role for Rab3D in preventing granule fusion.	Blank et al. (2002), Pombo et al. (2001), Roa et al. (1997), Riedel et al. (2002), Tuvim et al. (1999)
	Rab27a	+	+	KO+	Cytoplasmic, SG?	Might operate the transition from microtubule-driven granule mobility to actin-based exocytosis.	Goishi et al. (2004), Mizuno et al. (2007)
	Rab27b	+	+	KO+	Cytoplasmic, SG?	Double KO and to a lesser extent single KO animals display impaired passive cutaneous anaphylaxis responses. Bone marrow-derived MC from double/single KO animals show a decrease in antigen-induced histamine release.	
	Munc18-1	+	−	KO + (lethal)	ND		Nigam et al. (2005)
	Munc18-2	+	+	Human: FHL-5	SG	Negative regulators of SNARE assembly through binding to non-complexed syntaxins. Fusion promoters through binding to trans-SNARE complexes. Relocation to lamellipodia. Overexpression inhibits exocytosis in activated MC.	Martin-Verdeaux et al. (2003), Pombo et al. (2003), Tadokoro et al. (2007)

(Continued)

Table 1 | Continued

Group	Name	mRNA	Protein expression	KO model; human deficiency	Subcellular location in mast cells	Functional evidence for a role in mast cell exocytosis	Reference
	Munc18-3	+	+	KO + (lethal)	PM	Overexpression does not alter MC exocytosis	Martin-Verdeaux et al. (2003)
	Munc13-4	+	+	Human: FHL3	SG	Positive regulator of MC degranulation. Overexpression enhances activated MC exocytosis.	Neeft et al. (2005)
	SCAMP-1, -2, -3	ND	+		SG, PM	Colocalization at PM between SCAMPs, STX4, and SNAP-23. SCAMPs may serve as targets for SNAP-23 relocation, then contribute to the formation of the fusion pore.	Castle et al. (2002)
	Complexin II	+	+	KO+	Cytoplasmic	Relocation to PM in activated MC. Complexin II siRNA inhibits exocytosis. Interacts with a SNARE complex comprising SNAP-23, STX3, and VAMP2 or VAMP8. Direct binding to STX3 and to VAMP8 but not to SNAP-23.	Tadokoro et al. (2005, 2010), Takahashi et al. (1999)
	Synaptotagmin I	−	−		SG (exogenous)	Exogenously expressed synaptotagmin I enhances calcium ionophore-activated MC exocytosis.	Baram et al. (1998), Kimura et al. (2001)
	Synaptotagmin II	+	+	KO+	Lysosomes	Most abundant MC synaptotagmin. Required for degranulation and cell surface expression of MHC class II molecules. Synaptotagmin II siRNA enhances fusion of genuine lysosomes with SG. Synaptotagmin II KO mice: impaired passive cutaneous anaphylaxis, impaired MC degranulation.	Baram et al. (1999), Melicoff et al. (2009), Nagai et al. (2011), Peng et al. (2002)
	Synaptotagmin III	+	+		Early endosomes, SG	Synaptotagmin III antisense cDNA treated RBL cells display impaired function of the endocytic recycling compartment and enlarged SG size	Grimberg et al. (2003)
	Synaptotagmin IX	+	+		Endocytic recycling compartment, PM	Protein export from the endocytic recycling compartment toward PM. Sorting of SG proteins. Synaptotagmin IX siRNA slows down protein export from the endocytic recycling compartment to the cell surface.	Haberman et al. (2003, 2007)
	NCS-1	+	+			Stimulation of Fc epsilon receptor (through enhanced endocytic recycling) but not calcium ionophore induced exocytosis.	Kapp-Barnea et al. (2003, 2006)

Abbreviations: FHL, familial human lymphohistiocytosis; KO, knock-out; ND, not determined; MC, mast cells; NCS, neuronal calcium sensor; PM, plasma membrane; SG, secretory granules; STX, syntaxin.

SNAP-23. Rak3D was calcium-regulated as stimulation promoted the disappearance of the activity in Rab3D immunoprecipitates.

Taken together, the SNARE fusion machinery is tightly coupled to cell signaling. This coupling enables the activation, the fine tuning as well as the arrest of fusion events in a tightly regulated manner, which is necessary for appropriate secretory responses. It also integrates the signals from many different receptors able to induce stimulus-secretion coupling.

CONCLUDING REMARKS

It has now become clear that mast cells use a highly sophisticated machinery of proteins that enable the fusion of vesicles containing pre-stored and newly synthesized inflammatory mediators (**Figure 5**). Central to this machinery are SNARE proteins able to assemble into a tetrameric complex (**Figure 2**) that catalyzes the fusion of membranes of different cellular compartments. Although some of the central SNARE proteins involved in mast cell secretory events have been defined, gaps still persist in our understanding of the membrane fusion process. In particular, functional studies showed that the absence of one SNARE protein does not necessarily result in a complete block of exocytosis suggesting that some of its components are redundant. This is also supported by data showing that human mast cells use several types of v-SNARE proteins and that quality and quantity of detectable SNARE complexes is altered in knock-out cells. In addition, it has been known for many years that mast cells undergo compound exocytosis implying also fusion between granules. Although this could imply relocalization of plasma membrane SNAREs into the cell interior it cannot be excluded that different types of SNARE complexes are involved in this process. In agreement, preliminary data in our lab suggest that besides STX4, granule-localized STX3 may also play a role in the fusion process. Furthermore, little is still known about the vesicular carriers involved in chemokine/cytokine secretion (**Figure 3**). Recent data suggest a highly complex profile of vesicular carriers and involved SNARE proteins that may not follow a standardized scheme. In addition to the core fusion proteins, mast cells also express a series of accessory molecules that regulate SNARE assembly. These may act as "gatekeepers" of the fusion reaction holding effectors in an inactive state or by coupling the fusion machinery to cell signaling in order to fine tune the transition to a fusion competent state. Given the potentially dangerous nature of an inappropriate activation of this machinery, the development of hierarchically organized regulatory levels is advantageous. Thus, besides effectors that directly trigger the fusion reaction, proteins involved in granule transport, granule docking and priming have co-evolved to provide multiple layers of regulation. Still, little is known, of how these proteins are related to early signaling events, although some basic connections, such as the phosphorylation of SNAP-23 by IKK and the responsiveness of certain processes to calcium signals have been described.

The understanding of the molecular machinery has important therapeutic potential. Indeed, it has become clear that during a generalized inflammatory reaction, multiple receptors and pathways are involved, many of these converge to trigger the secretion of inflammatory mediators. Thus, targeting the late fusion machinery clearly represents a strategy able to interfere with the activation of multiple receptors and limit their effect on mediator secretion.

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The role of TRP proteins in mast cells

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Transient receptor potential (TRP) proteins form cation channels that are regulated through strikingly diverse mechanisms including multiple cell surface receptors, changes in temperature, in pH and osmolarity, in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), and by phosphoinositides which makes them polymodal sensors for fine tuning of many cellular and systemic processes in the body. The 28 TRP proteins identified in mammals are classified into six subfamilies: TRPC, TRPV, TRPM, TRPA, TRPML, and TRPP. When activated, they contribute to cell depolarization and Ca^{2+} entry. In mast cells, the increase of $[Ca^{2+}]_i$ is fundamental for their biological activity, and several entry pathways for Ca^{2+} and other cations were described including Ca^{2+} release activated Ca^{2+} (CRAC) channels. Like in other non-excitable cells, TRP channels could directly contribute to Ca^{2+} influx via the plasma membrane as constituents of Ca^{2+} conducting channel complexes or indirectly by shifting the membrane potential and regulation of the driving force for Ca^{2+} entry through independent Ca^{2+} entry channels. Here, we summarize the current knowledge about the expression of individual Trp genes with the majority of the 28 members being yet identified in different mast cell models, and we highlight mechanisms how they can regulate mast cell functions. Since specific agonists or blockers are still lacking for most members of the TRP family, studies to unravel their function and activation mode still rely on experiments using genetic approaches and transgenic animals. RNAi approaches suggest a functional role for TRPC1, TRPC5, and TRPM7 in mast cell derived cell lines or primary mast cells, and studies using Trp gene knock-out mice reveal a critical role for TRPM4 in mast cell activation and for mast cell mediated cutaneous anaphylaxis, whereas a direct role of cold- and menthol-activated TRPM8 channels seems to be unlikely for the development of cold urticaria at least in mice.

Keywords: TRP proteins, cation channels, Ca^{2+} signaling, mast cell activation

CA^{2+} ENTRY PATHWAYS AND MAST CELL ACTIVATION

In mast cells, the increase of free cytosolic Ca^{2+} regulates a variety of cellular processes including degranulation with release of preformed inflammatory mediators (Ozawa et al., 1993), production of eicosanoids such as leukotrienes (Chang et al., 2006), activation of transcription factors including the nuclear factor of activated T cells (NFAT; Kar et al., 2011), and synthesis of cytokines (Plaut et al., 1989) as well as cytoskeletal rearrangements required for migration of mast cells and chemotaxis (Hartmann et al., 1997). Mast cell derived mediators can evoke both pathological inflammatory responses in allergic or autoimmune diseases but they can also have protective functions, e.g., by induction of innate immune responses leading to clearance of pathogens or by degrading endogenous and exogenous toxins (Marshall, 2004; Galli et al., 2005; Metz and Maurer, 2007). The importance of extracellular Ca^{2+} as a requirement for anaphylactic release of histamine from rat peritoneal mast cells was already shown in 1972 (Foreman and Mongar, 1972), and it could be shown that Ca^{2+} could be replaced by Sr^{2+} or Ba^{2+} to achieve mast cell degranulation (Foreman et al., 1977). Later, it was shown that substantial amounts of Ca^{2+} in the external medium (i.e., $>50\,\mu M$) was absolutely required for degranulation in rat basophilic leukemia (RBL)-2H3 cells (Beaven et al., 1984) leading to the concept that antigen-mediated mast cells degranulation is dependent on Ca^{2+} influx through Ca^{2+} channels that are permeable for Sr^{2+}/Ba^{2+} (Ma and Beaven, 2009, 2011). Distinct temporal and spatial patterns of the increase in intracellular Ca^{2+} concentration triggered by individual Ca^{2+} mobilizing mast cell activators may explain the specificity of mast cell responses. For example, differences in the amplitude and duration of Ca^{2+} signals in response to different stimulants differentially influence histamine secretion or production of eicosanoids (van Haaster et al., 1995). Also, it was shown that, under certain conditions, mast cells can be stimulated to undergo chemotaxis without degranulation (Taub et al., 1995). More recent work has revealed that a range of mast cell responses are activated by spatially restricted Ca^{2+} signals just below the plasma membrane (Di Capite and Parekh, 2009; Kar et al., 2011).

Although mast cells have numerous functions beyond the development of allergies, studies of mast cell signal transduction have mainly been driven by the central role of these cells in allergic inflammatory responses (Metcalfe et al., 1997). The manifestations of mast cell-driven allergic reactions are considered to be mainly a consequence of the release of pro-inflammatory mediators following antigen-induced aggregation of high-affinity receptors for IgE (FcεRI), expressed at the mast cell surface. FcεRI cross-linking activates a large number of signaling molecules (Blank and Rivera,

2004; Gilfillan and Tkaczyk, 2006). A major downstream target is phospholipase Cγ1 (PLCγ1), which catalyzes the hydrolysis of phosphatidylinositol (4,5) bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP_3). DAG and IP_3 promote protein kinase C (PKC) activation and release of Ca^{2+} from intracellular stores, respectively, followed by an influx of Ca^{2+} from the extracellular space. Also in other non-excitable cells the depletion of intracellular Ca^{2+} stores correlates with influx of Ca^{2+} from the extracellular space suggesting that the amount of Ca^{2+} in the stores controls the extent of Ca^{2+} influx, a process that was called store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+} entry (CCE; Putney, 1986; Parekh and Putney, 2005) and described later also in mast cells (Putney, 1986; Ali et al., 1994; Falcone and Fewtrell, 1995). Ionic currents mediating this Ca^{2+} influx were first characterized by Hoth and Penner (1992, 1993) in RBL cells and rat peritoneal mast cells. The physiological hallmark of the underlying channel is a high selectivity for Ca^{2+} over other cations and its activation by depletion of intracellular Ca^{2+} stores, e.g., by IP_3 and chelation of cytosolic Ca^{2+}. The Ca^{2+} selectivity of Ca^{2+} release activated Ca^{2+} (CRAC) channels is similar to that of voltage-activated Ca^{2+} channels (VDCC) which were shown to be expressed and to modulate mast cell activation (Yoshimaru et al., 2009), but the conductance of CRAC channels is much lower. Also, replacement of external Ca^{2+} with Sr^{2+} or Ba^{2+} resulted in a decline in I_{CRAC} activity (Zweifach and Lewis, 1996) indicating that CRAC channels cannot represent the exclusive Ca^{2+} entry channel mediating Ca^{2+} dependent mast cell degranulation. The desire to identify the molecular nature of SOCE was and still is the motivation for many workers to characterize mammalian transient receptor potential (TRP) channels. However, the pore properties of most TRP channels that have been studied in detail, including TRPV6, which was identified in primary murine and human mast cells (Turner et al., 2007), and TRPV5, appeared not to match the pore properties of CRAC channels (see below and Owsianik et al., 2006b). Recently, Orai1 (also known as CRACM1) was identified as the pore-forming CRAC channel subunit (Feske et al., 2006; Vig et al., 2006; Lewis, 2007) and another protein called stromal interaction molecule 1 (Stim1) was shown to represent the Ca^{2+} sensor coupling the process of depletion of intracellular Ca^{2+} stores with Ca^{2+} influx across the plasma membrane through CRAC channels (Lewis, 2007). Orai1 and its homologs Orai2 and Orai3 were shown to be expressed in RBL-2H3 cells and bone marrow-derived mast cells (BMMCs; Gross et al., 2007). SOCE is substantially decreased in *Orai1*-deficient BMMCs (Gwack et al., 2008), and the FcεRI-mediated Ca^{2+} entry is reduced by 70%, while the cells completely lack detectable Ca^{2+} (CRAC) currents (Vig et al., 2008). Interestingly, in this study it was concluded that Ca^{2+} release from intracellular stores is unchanged in *Orai1*-deficient BMMCs (Vig et al., 2008) suggesting that ORAI1 proteins are non-critical components for the refilling of these Ca^{2+} stores. The relative contribution of Orai2 and Orai3 for Ca^{2+} entry in mast cells is still unclear. Although these results support the conclusion that ORAI1 (CRACM1)-proteins build the Ca^{2+}-selective channels responsible for the majority of Ca^{2+} entry into mast cells, there is still some residual Ca^{2+} entry in *Orai1*-deficient BMMCs after stimulation of the FcεRI or store depletion. Ca^{2+} entry in *Orai1*-deficient mast cells following activation with other Ca^{2+} mobilizing mast cell activators has not been analysed so far. This suggests that other channel proteins – especially members of the TRP family such as TRPC5 (Ma et al., 2008) – might contribute to Ca^{2+} entry either as part of an Orai/TRP channel complex (Liu et al., 2003, 2007; Liao et al., 2007; Yuan et al., 2007) or independently.

In addition to activation of FcεRI and depletion of intracellular Ca^{2+} stores there is increasing evidence that receptors for other ligands such as adenosine, endothelin-1, stem cell factor (SCF), lysophosphatidylcholine (LPC), sphingosine-1 phosphate (S1P), substance P, and others, which markedly influence mast cell activation in a physiological setting, do also induce elevations of $[Ca^{2+}]_i$. These receptors can either potentiate FcεRI-mediated mast cell activation or, by themselves, stimulate the release of mast cell mediators. The pathways leading to elevation of $[Ca^{2+}]_i$ following stimulation of these receptors are still poorly characterized, and particularly it is not known whether or to which degree the resultant receptor-activated Ca^{2+} entry is mediated by store depletion. Receptor stimulation using substance P or compound 48/80 leads to a Ca^{2+} influx through non-selective cation channels in rat peritoneal mast cells (Penner et al., 1988; Kuno and Kimura, 1992; Fasolato et al., 1993). Recently, an additional mechanism for regulation of Ca^{2+} entry in mast cells has been discovered that does not rely on mast cell intrinsic mechanisms such as receptor stimulation by soluble mediators or regulation of the driving force for Ca^{2+} entry but on the interaction with another cell type: it could be shown that binding of OX40 expressed on regulatory T cells to the mast cell based receptor OX40L inhibits Ca^{2+} entry by an increase of intracellular cAMP levels in mast cells (Gri et al., 2008). The molecular constituents of the Ca^{2+} conducting channels regulated by this new pathway are unknown like those channels activated by the numerous Ca^{2+} mobilizing agonists described above, but members of the TRP family are potential candidates also for these Ca^{2+} entry pathways.

TRP PROTEINS FORM CATION CHANNELS

Transient receptor potential channels are a large and functionally heterogeneous family of cation-conducting channel proteins, which are activated and regulated through strikingly diverse mechanisms. The first TRP channel gene was discovered in *Drosophila melanogaster* (Montell and Rubin, 1989) in the analysis of a fly mutant whose photoreceptors failed to retain a sustained response to maintained light stimuli. In mammals, TRP proteins were identified in most cases by their sequence homology. Nevertheless, some TRPs were identified by expression cloning, e.g., TRPV1 as the receptor for vanilloids such as capsaicin, or by positional cloning efforts to identify genes disrupted in human diseases, e.g., TRPML1 (also designated as mucolipin, MCOLN1, ML1) as a gene that is mutated in mucolipidosis type IV or TRPP2 (also designated as polycystin-2, PKD2, PC2) in autosomal polycystic kidney disease.

The mammalian 28 TRP proteins are classified according to structural homology into six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin), and TRPP (polycystin; Montell et al., 2002; Clapham et al., 2005; Wu et al., 2010). All TRP channels are assumed to have six-transmembrane (6TM) polypeptide subunits that assemble as tetramers to form cation-permeable pores (Owsianik et al., 2006a).

Most TRP channels show little voltage dependence and are non-selective with a permeability for Ca^{2+} over Na^{+} (ratio P_{Ca}/P_{Na}) below 10. Therefore, TRP channels are not only important for Ca^{2+} entry via the plasma membrane but play also an important role in electrogenesis regulating the driving forces for Ca^{2+} entry via other Ca^{2+}-permeable channels. **Table 1** (Venkatachalam and Montell, 2007; Gees et al., 2010) gives an overview about the permeability of channels formed by individual TRP proteins, but it has to be emphasized that most of these information is based on studies of heterologously expressed channel proteins and the characteristics of those channels may differ significantly from native channel complexes existing in primary cells since the ectopically expressed channel proteins do not necessarily act in accordance with the native cellular context as in primary cells.

Transient receptor potentials are expressed in many (if not in all) excitable and non-excitable cells and are involved in sensing of a variety of environmental stimuli such as temperature, pH, osmolarity, pheromones, taste, and plant compounds. TRP channels participate in numerous cellular processes to determine organ and integrative body functions, and several mutations in Trp genes appear to be causative factors in rare heritable channelopathies (Freichel and Flockerzi, 2007; Nilius and Owsianik, 2010). Although the search for natural ligands and chemical modulators of TRP channels as therapeutics has been intensified in the last years, specific agonists or blockers are still lacking for most members of the TRP family until now. Therefore, genetic approaches are still required to advance a causal understanding of their physiological functions in primary cells, in organs, for systemic functions of organisms and for disease states. These approaches include over-expression of dominant-negative variants, antisense oligonucleotides, and RNAi as well as targeted deletion of the gene of interest using homologous recombination (Freichel et al., 2011). Another obstacle that hinders the analysis of endogenous TRP channels is that specific antibodies are rare for most TRP proteins (Meissner et al., 2011) and, accordingly, this impedes investigations of the assembly and localization of TRP channels, but also the control of the effectiveness of RNAi approaches.

EXPRESSION AND POSTULATED FUNCTIONS OF TRP PROTEINS IN MAST CELLS

In the following we highlight key features regarding activation and functions of individual members of the TRP subfamilies, concerning their expression in mast cell models and established or postulated mast cell functions. **Table 1** systematically summarizes information about the permeability, mode of activation, and the biological functions in native systems as revealed by mouse models for the individual mammalian TRP proteins. For more detailed information regarding structure, gating, special functional aspects of splice variants, TRP channelopathies, and citations of the wealth of original manuscripts about mammalian TRP proteins since their first description and functional characterization in 1995/1996 (Wes et al., 1995; Zhu et al., 1995; Philipp et al., 1996), we refer to various excellent recent review articles (Venkatachalam and Montell, 2007; Gees et al., 2010; Nilius and Owsianik, 2010; Wu et al., 2010). The current knowledge about the expression of TRP proteins in different cell lines and primary mast cell models (**Table 2**) is based on analysis using RT-PCR, gene arrays or quantitative PCR approaches as well as Western Blot analysis or immunocytochemistry for which commercial anti-TRP antibodies were used in most cases. For the latter approach, it has to be mentioned that many commercial suppliers pass the burden of antibody validation to the end user and that the specificity of the antibodies used was rarely tested rigorously in preparations of cells/tissues that do not express the target protein at all (Meissner et al., 2011). The functional role of individual TRP proteins in mast cells was analysed using TRP channel antagonists, RNAi approaches, and TRP deficient mouse models. Regarding RNAi approaches it needs to be noted that – despite the merits of this technology – off-target effects are not unusual, and were also described in a study where transfection of a *Trpm7*-specific siRNA achieved complete suppression of *Trpm7* mRNA in other primary cells but also significantly reduced TRPM2 expression levels (Aarts et al., 2003). Secondly, the control of the effectiveness of RNAi approaches requires specific antibodies against the target TRP protein to avoid the situation that at the same time a poorly characterized RNAi experiment is controlled by non-validated antibodies and vice versa. Reports using mast cells derived from mice with targeted deletion of Trp genes are restricted until now to cells from mice with ubiquitous inactivation of individual Trp genes, in which the observed phenotype may be affected by compensatory mechanisms.

TRPC CHANNELS

The mammalian members of the TRPC family can be divided into three subfamilies on the basis of functional similarities and sequence homology: TRPC1/TRPC4/TRPC5, TRPC3/TRPC6/TRPC7, and TRPC2. Physical interaction between the TRPC family members was studied by co-immunoprecipitation and FRET experiments with heterologously expressed proteins and using immunoprecipitation from brain protein fraction using antibodies directed against peptides derived from TRPC proteins and revealed that TRPC1, TRPC4, and TRPC5 may coassemble as well as TRPC3, TRPC6, and TRPC7 (Lintschinger et al., 2000; Strubing et al., 2001; Goel et al., 2002; Hofmann et al., 2002). In embryonic rat brain, it was found that TRPC1, TRPC4, and TRPC5 interact with TRPC3 and TRPC6 (Strubing et al., 2003). All TRPC proteins seem to be activated by stimulation of G-protein-coupled receptors or receptor-tyrosine kinases. The activation of the PLC pathway leads to the opening of TRPC channels but in parallel to stimulation of IP_3 receptors and subsequent depletion of intracellular Ca^{2+} stores and, hence, SOCE. This raised the issue whether TRPC proteins are constituents of cation channels mediating SOCE, and a contribution of TRPC proteins to SOCE has been suggested very early after their first discovery (Hardie and Minke, 1993). In principle, over-expressed mammalian TRPCs have all been reported to be activated by store depletion (for review see, e.g., Venkatachalam et al., 2002). However, the dependence of TRPCs on store depletion requires special conditions. For instance, TRPC3 channels, which are activated directly by PLC-derived DAG in many expression systems, were also reported to be activated by reduction of the filling state of intracellular Ca^{2+} stores (Kiselyov et al., 1998; Vazquez et al., 2003), but the ability of TRPC3 to contribute to SOCE was only found when it was expressed at low densities (Vazquez et al., 2003; Yildirim et al., 2005). The underlying cause for this phenomenon has not

Table 1 | Properties of channels formed by mammalian TRP proteins.

Subtype	Selectivity P_{Ca2+}/P_{Na+}	Activation/modulation of activity	Consequences of TRP-deletion in mice
TRPC1	∼1	PLC activation, store depletion, conformational coupling, mechanical stretch	Elevated body weight, impaired salivary gland fluid secretion
TRPC2	∼1–3	PLC activation, diacylglycerol (DAG)	Abnormal sexual and mating behavior
TRPC3	∼1.5	PLC activation, store depletion, conformational coupling, DAG, exocytosis	Defects in motor coordination and walking behavior
TRPC4	∼1–8	PLC activation, store depletion (?), PIP_2 breakdown, exocytosis	Impaired vascular function, altered 5-HT-mediated GABA release, defects in intestinal motility
TRPC5	∼2–9	PLC activation, store depletion (?), sphingosine-1-phosphate, exocytosis	Decreased anxiety-like behavior
TRPC6	∼5	PLC activation, conformational coupling, DAG, PIP_3	Grossly normal, increased artery contractility, impaired light response in intrinsically photosensitive retinal ganglion cells in TRPC6/TRPC7 compound KO mice
TRPC7	∼1–5	PLC activation, store depletion, DAG	Impaired light response in intrinsically photosensitive retinal ganglion cells in TRPC6/TRPC7 compound KO mice
TRPV1	∼4–10	Heat (43˚C), vanilloids, proinflammatory cytokines, protons, PIP_2	Reduced inflammatory hyperalgesia, impaired bladder function
TRPV2	∼1–3	Heat (52˚C), osmotic cell swelling, exocytosis	Accelerated mortality in bacterial infection
TRPV3	∼1–10	Warm (33–39˚C); PUFAs; menthol; compounds from oregano, cloves, and thymes	Impaired thermosensation, skin barrier effects, curved whiskers, and hair
TRPV4	∼6	Warm (27–34˚C), osmotic cell swelling, 5′ 6′-EET, anandamide, 4αPDD, exocytosis	Altered body osmolarity; increased bone mass; impaired bladder function; reduced inflammatory hyperalgesia
TRPV5	>100	Constitutively active[1], exocytosis (?)	Impaired renal Ca^{2+} reabsorption; decreased bone thickness
TRPV6	>100	Constitutively active[1], store depletion (?), exocytosis (?)	Impaired epididymal Ca^{2+} absorption, male hypofertility, impaired Ca^{2+} absorption
TRPM1	<1	Translocation (?)	Impaired ON bipolar cell function and vision
TRPM2	∼0, 3–2	ADP-ribose, cADP-ribose, pyrimidine nucleotides, arachidonic acid, NAD, H_2O_2, Ca^{2+}	Impaired neutrophil infiltration in inflammation, increased ROS production in phagocytes
TRPM3	$\sim 1–10^2$	constitutively active[1], osmotic cell swelling, store depletion (?), d-erythro-sphingosine (?), pregnenolone sulfate	Impaired noxious heat perception
TRPM4	Monovalent selective	Ca^{2+}, voltage modulated, PIP_2	Increased release of inflammatory mediators from mast cell and cutaneous anaphylaxis; impaired dendritic cell migration; reduced secondary hemorrhage and lesions after spinal cord injury, hypertension associated with increased catecholamine release from chromaffin cells
TRPM5	Monovalent selective	Taste receptor activation (T1R, T2R), Ca^{2+}, voltage modulated, PIP_2, heat (15–35˚C)	Impaired sweet, umami, and bitter taste reception; deflects in glucose-induced insulin release
TRPM6	$<10^3$	Mg^{2+} inhibited, translocation	Embryonic lethality, neural tube defects in development
TRPM7	$\sim 0.2–2^3$	Activation mode of native channels unclear, Mg^{2+} inhibited, ATP, protons, phosphorylation, PIP_2	Embryonic lethality; conditional TRPM7 deletion in T cells causes abnormal thymocyte development
TRPM8	∼0.3–4	Cool (23–28˚C), menthol, icilin, pH modulated, PIP_2	Deficiencies in response to cold
TRPA1	∼0.8–5	Cold (17˚C) (?), icilin, isothiocyanates, allicin (garlic), cannabinoids, bradykinin, PLC activation, DAG, PUFAs	Reduced response to noxious cold and intestine mechanical force

(Continued)

Table 1 | Continued

Subtype	Selectivity P_{Ca2+}/P_{Na+}	Activation/modulation of activity	Consequences of TRP-deletion in mice
TRPML 1	[4]	Activation mode of native channels unclear, potentiation by low pH	Motor deficits, retinal degeneration, decreased life span
TRPML 2	[4]	Activation mode of native channels unclear, potentiation by low pH	–
TRPML 3	[5]	Activation mode of native channels unclear, removal, and readdition of extracellular Na^+	Varitint-waddler (Va) mice with a TRPML3 (A419P) gain of function mutation exhibit deafness, circling behavior, and pigmentation defects
TRPP2	Non-selective	Translocation with TRPP1, fluid flow, mechanical gating (?)	Lethal E13; embryonic cysts and extrarenal abnormalities including left-right asymmetry of visceral organs
TRPP3	∼1–10	Ca^{2+}, voltage modulated	–
TRPP5	non-selective	?	–

[1]Not yet measured in primary cells; [2]significant differences in individual splice variants; [3]divalent cation selective (Ca^{2+} and Mg^{2+}); [4]localization primarily intracellularly in endolysosomes, permeability for Na^+, K^+, Ca^{2+}, Fe^{2+}, for more details see Cheng et al. (2010); [5]localization primarily intracellularly in endolysosomes, permeability for Na^+, Ca^{2+}, K^+; for more details see Cheng et al. (2010).

been identified, but one explanation is that at high expression levels the transfected TRPCs titrate regulatory subunits that confer SOCE characteristics to TRPCs. Although numerous studies show that Orai1 is the pore-forming subunit of store-operated channels (Lewis, 2007), a model with Orai as a regulatory subunit of SOCE channels composed of pore-forming TRPC subunits has recently been proposed (Liao et al., 2008). Additionally, Stim1 was found to hetermultimerize TRPC proteins either directly (TRPC1, TRPC4, TRPC5) or indirectly (TRPC3, TRPC6) to determine their store-operated activation mode (Yuan et al., 2007, 2009). Taken together, the question of whether and how TRPC proteins contribute to SOCE remains highly controversial (Parekh, 2010) and cannot be generally answered but may depend on the relative expression of the above mentioned and/or not yet identified constituents that make up SOC channel complexes in individual cell types.

The permeability (P_{Ca}/P_{Na}) of channels formed by heterologously expressed TRPC proteins varies between 1 and 10 (**Table 1** and Venkatachalam and Montell, 2007; Gees et al., 2010). Whether TRPC1 can form functional homomeric channels by itself still remains debatable. TRPC1 forms heteromeric channels with TRPC4 or TRPC5, which have properties distinct from those of homomultimers (Strubing et al., 2001). TRPC4 and TRPC5 share many structural and functional characteristics such as the activation by $G_{q/11}$-coupled receptors. On the other hand there are considerable discrepancies regarding the precise activation mechanism in response to PLC stimulation and with respect to their channel properties (e.g., permeability), even if homomeric TRPC proteins are analyzed (Cavalie, 2007). The differences may result from the expression system used and thereby emphasize the need to analyze the action of individual TRPC proteins in the context of their native environment. This can be exemplified by studies in mice with inactivation of the *Trpc4* gene. Depending on the cell type studied, channels with completely different characteristics are impaired in *Trpc4*-deficient mice: deletion of the *Trpc4* gene results in 95% reduction of acetylcholine (ACh)-triggered store-operated channels with a Ca^{2+} over Na^+ selectivity of >100 in endothelial cells whereas in ileal smooth muscle cells the loss of TRPC4 leads to an 80% reduction of ACh-evoked currents which are predominantly carried by Na^+ and activated by PIP_2 breakdown rather than by store depletion (Freichel et al., 2001; Tsvilovskyy et al., 2009). These results show that TRPC gating and function cannot necessarily be extrapolated across cell types.

TRPC3, TRPC6, and TRPC7 when expressed in heterologous systems are potentiated by $G_{q/11}$-coupled receptors or by direct application of diacylglycerol (DAG) analogs (Hofmann et al., 1999). Interestingly, the density of inward currents evoked by a DAG derivate were significantly higher in smooth muscle cells isolated from *Trpc6*$^{-/-}$ mice. This was explained by formation of TRPC3 homo-oligomeric channel complexes in TRPC6-deficient smooth muscle cells, because mRNA expression of *Trpc3* appears to be up-regulated two- to threefold in cells of *Trpc6*$^{-/-}$ mice (Dietrich et al., 2005). This example demonstrates that the analysis of cation channels consisting of TRPC proteins is further aggravated by the fact that inactivation of a given TRP protein may be compensated by up- or down-regulation of other genes including physically or functionally interacting TRP genes. Time-dependent inactivation strategies may be used in such cases, or compound knock-out mice in which all redundant TRP proteins are inactivated simultaneously.

Trpc2 is a pseudogene in humans, but its rodent ortholog encodes a functional TRPC2 channel important to pheromone sensing in vomeronasal organ, where it can be directly activated by DAG (Lucas et al., 2003).

Expression of *Trpc* genes was found in various types of mast cells (**Table 2**). TRPC1 has been detected in murine BMMC on mRNA (Sanchez-Miranda et al., 2010; Suzuki et al., 2010) and protein (Hernandez-Hansen et al., 2004) level, also in the rat cell line RBL-2H3 (Ma et al., 2008) and using a microarray expression analysis in human skin mast cells (Bradding et al., 2003). In RBL-2H3 cells, knockdown of TRPC1 and TRPC3 proteins through expression of corresponding shRNAs decreased the cells sensitivity to antigen-stimulation and shifted the Ca^{2+} wave initiation site from the tips of extended cell protrusions to the cell body (Cohen et al., 2009). Interestingly, in mice deficient for the Src

Table 2 | Expression of TRP transcripts and proteins in mast cell models.

Species	Cell type	Name	Method	Reference
TRPC1				
Mouse	Primary cells	BMMC	qRT-PCR	Suzuki et al. (2010)
Mouse	Primary cells	BMMC	RT-PCR	Sanchez-Miranda et al. (2010)
Rat	Cell line	RBL-2H3	RT-PCR, WB	Ma et al. (2008)
Mouse	Primary cells	BMMC	WB	Hernandez-Hansen et al. (2004)
Human	Primary cells	skin mast cells	Affymetrix gene array	Bradding et al. (2003)
TRPC2				
Rat	Cell line	RBL-2H3	RT-PCR	Ma et al. (2008)
TRPC3				
Mouse	Primary cells	BMMC	RT-PCR	Sanchez-Miranda et al. (2010)
Mouse	Primary cells	L138.8A	qRT-PCR	Sel et al. (2008)
Rat	Cell line	RBL-2H3	RT-PCR, WB	Ma et al. (2008)
Mouse	Primary cells	BMMC	WB	Hernandez-Hansen et al. (2004)
TRPC4				
Mouse	Primary cells	BMMC	qRT-PCR	Suzuki et al. (2010)
Mouse	Primary cells	BMMC	WB	Hernandez-Hansen et al. (2004)
TRPC5				
Mouse	Primary cells	BMMC	qRT-PCR	Suzuki et al. (2010)
Mouse	Primary cells	BMMC	RT-PCR	Sanchez-Miranda et al. (2010)
Rat	Cell line	RBL-2H3	RT-PCR, WB	Ma et al. (2008)
TRPC6				
Mouse	Primary cells	BMMC	RT-PCR	Sanchez-Miranda et al. (2010)
Mouse	Primary cells	BMMC	WB	Hernandez-Hansen et al. (2004)
Mouse	Cell line	PB-3c	NB	Buess et al. (1999)
TRPC7				
Rat	Cell line	RBL-2H3	RT-PCR	Ma et al. (2008)
Mouse	Primary cells	BMMC	IP	Sanchez-Miranda et al. (2010)
TRPV1				
Human	Cell line	HMC-1	WB, RT-PCR	Zhang et al. (2011)
Human	Mast cells in bladder		IHC	Lazzeri et al. (2004)
Rat	Cell line	RBL-2H3	RT-PCR	Stokes et al. (2004)
Human	Skin mast cells		IHC	Stander et al. (2004)
TRPV2				
Human	Cell line	HMC-1	WB, RT-PCR	Zhang et al. (2011)
Human	Cell line	HMC-1	RT-PCR	Kim et al. (2010)
Rat	Cell line	RBL-2H3	RT-PCR, NB, WB, ICC, qPCR	Stokes et al. (2004)
Mouse	Cell line	P815	NB, WB	Stokes et al. (2004)
Mouse	Primary cells	BMMC	WB, (qPCR)	Stokes et al. (2004)
Rat	Cell line	RBL-2H3	NB, WB, ICC	Stokes et al. (2005)
Mouse	Cell line	P815	NB, WB	Stokes et al. (2005)
Human	Primary cells	HLMC	Affymetrix gene array	Bradding et al. (2003)
Human	Primary cells	Skin mast cells	Affymetrix gene array	Bradding et al. (2003)
Human	Primary cells	CBMC	Affymetrix gene array	Bradding et al. (2003)
TRPV3				
Not reported				
TRPV4				
Human	Cell line	HMC-1	WB, RT-PCR	Zhang et al. (2011)
Human	Cell line	HMC-1	RT-PCR	Kim et al. (2010)
Rat	Cell line	RBL-2H3	ICC	Yang et al. (2007)
Rat	Cell line	RBL-2H3	RT-PCR	Stokes et al. (2004)
TRPV5				
Not reported				

(Continued)

Table 2 | Continued

Species	Cell type	Name	Method	Reference
TRPV6				
Rat	Cell line	RBL-2H3	RT-PCR	Stokes et al. (2004)
TRPM1				
		Not reported		
TRPM2				
Human	Primary cells	HLMC	Affymetrix gene array	Bradding et al. (2003)
Human	Primary cells	CBMC	Affymetrix gene array	Bradding et al. (2003)
TRPM3				
		Not reported		
TRPM4				
Mouse	Primary cells	BMMC	RT-PCR, NB, WB, ICC	Vennekens et al. (2007)
TRPM5				
		Not reported		
TRPM6				
		Not reported		
TRPM7				
Rat	Cell line	RBL-2H3	RT-PCR	Stokes et al. (2004)
Human	Primary cells	HLMC	RT-PCR	Wykes et al. (2007)
Human	Cell line	HMC-1	RT-PCR	Wykes et al. (2007)
Human	Cell line	LAD-2 cells	RT-PCR	Wykes et al. (2007)
TRPM8				
Mouse	Primary cells	BMMC	RT-PCR	Medic et al. (2011)
Rat	Cell line	RBL-2H3	RT-PCR, ICC	Cho et al. (2010)
TRPA1				
Rat	Cell line	RBL-2H3	ICC, WB	Prasad et al. (2008)
TRPP1				
		Not reported		
TRPP2				
		Not reported		
TRPP3				
		Not reported		
TRPML1				
		Not reported		
TRPML2				
		Not reported		
TRPML3				
		Not reported		

BMMC, bone marrow-derived mast cells; CMBC, cord blood derived mast cells; HLMC, human lung mast cells; ICC, immunocytochemistry; IHC, immunohistochemistry; IP, Immunoprecipitation; NB, Northern blot; (q)RT-PCR, (quantitative) reverse transcriptase polymerase chain reaction; WB, Western blot.

family kinase Fyn expression of TRPC1 proteins was reduced by ∼30%, and cation currents, depolymerization of cortical F-actin and degranulation triggered by FcεRI-stimulation was significantly reduced whereas mast cell degranulation evoked by ATP, substance P, or thrombin was unaffected. Similar effects were observed by downregulation of TRPC1 expression using siRNAs against TRPC1 and the deficits in FcεRI-triggered mast cell activation could be rescued by exogenous expression of TRPC1 (Suzuki et al., 2010). *Trpc2* mRNA has been reported in mouse BMMC using RT-PCR (Ma et al., 2008). Expression of TRPC3 was reported in mouse BMMC via Western Blot (Hernandez-Hansen et al., 2004) and RT-PCR (Sanchez-Miranda et al., 2010) and with both methods in the cell lines L138.8A and RBL-2H3 (Ma et al., 2008). In BMMCs, transcripts of *Trpc4* and *Trpc5* (Suzuki et al., 2010) and TRPC4 proteins (Hernandez-Hansen et al., 2004) were found. TRPC5 transcript and protein expression was reported in RBL-2H3 cells (Ma et al., 2008). shRNA-mediated knock down of TRPC5 in RBL-2H3 cells was associated with a reduced Ca^{2+} entry following depletion of intracellular Ca^{2+} stores and based on overexpression experiments in these cells it was proposed that TRPC5 associates with Stim1 and Orai1 in a stoichiometric manner to build Ca^{2+} and Sr^{2+} permeable channels that can be discriminated from channels made by Orai1 and Stim1 (Ma et al., 2008). However, receptor-mediated Ca^{2+} entry or membrane currents have not been analysed in this study. *Trpc6* and *Trpc7* transcripts and proteins were also found in mouse BMMC (Hernandez-Hansen

et al., 2004; Sanchez-Miranda et al., 2010) and based on immunoprecipitation experiments using an antibody that was designed to detect all three members of the TRPC3/TRPC6/TRPC7 subfamily it was proposed that TRPC3/TRPC6/TRPC7, like TRPC1 (Suzuki et al., 2010), interact with fyn kinase during FcεRI-mediated mast cell activation (Sanchez-Miranda et al., 2010).

TRPV CHANNELS

Similar to the TRPC family, the TRPV (vanilloid) family can be divided into two subfamilies on the basis of structure, function and Ca^{2+} selectivity: TRPV1-4, and TRPV5/6. In the TRPV subfamily, TRPV5 and TRPV6 can form heteromeric channel complexes (Hoenderop et al., 2003b; Hellwig et al., 2005; Schaefer, 2005). Furthermore, TRPV1 can associate with TRPV2 and TRPV3 (Cheng et al., 2007) and widespread interaction has been shown for TRPV1-TRPV4 (Cheng et al., 2007).

TRPV1, TRPV2, TRPV3, and TRPV4 are non-selective cation channels that are activated by a different range in temperatures, respectively, and by numerous other stimuli (**Table 1**). In addition, TRPV1 could be activated by low pH (Caterina et al., 1997; Jordt et al., 2000) and by vanilloid compounds, such as capsaicin and capsinate found in hot (chili) and non-pungent (bell) peppers, respectively (Caterina et al., 1997; Iida et al., 2003).

TRPV2 (like TRPV4) is activated by osmotic cell swelling and has a critical role in macrophage particle binding and phagocytosis (Link et al., 2010). TRPV3 is activated by a variety of botanical compounds (Moqrich et al., 2005). TRPV4 is sensitive to osmotic and mechanical stimuli, such as cell swelling or fluid flow. It could be activated by arachidonic acid metabolite 5′, 6′-epoxyeicosatrienoic acid (5′, 6′-EET; Vriens et al., 2005) or by 4α-Phorbol 12,13-Didecanoate (Watanabe et al., 2002).

TRPV5 and TRPV6 are the only highly Ca^{2+}-selective channels in the TRP channel family. They are not heat-sensitive and tend to be active at low $[Ca^{2+}]_i$ concentrations and physiological membrane potentials (Vennekens et al., 2000). Both proteins form constitutively active channels when heterologously expressed in different cell lines, but similar channel activity has never been recorded in the primary cell types that express TRPV5 or TRPV6, respectively. TRPV5 is essential for Ca^{2+} reabsorption in the kidney (Hoenderop et al., 2003a), and TRPV6 proteins determine Ca^{2+}-absorption in the epididymal epithelium and, thereby, sperm function and male fertility (Weissgerber et al., 2011).

All members of the TRPV proteins were reported to be expressed in various mast cell models except TRPV3 and TRPV5. For example, transcripts of *Trpv1*, *Trpv2*, *Trpv4*, and *Trpv6* were identified in RBL-2H3 cells (Stokes et al., 2004), *Trpv1*, *Trpv2*, and *Trpv6* in the human cell line HMC-1 (Zhang et al., 2011) and TRPV1 proteins in mast cells of the human bladder (Lazzeri et al., 2004) and skin (Stander et al., 2004). The TRPV1 specific agonists capsaicin or resiniferatoxin induced calcium uptake in several mouse mast cell lines, in BMMC, but not in PMC (Biro et al., 1998).

Treatment of BMMCs with ruthenium red, that is used as non-specific inhibitor of TRPV channels (Vriens et al., 2009), inhibited FcεeRI-mediated increase of cytosolic Ca^{2+} concentration (Lam et al., 2008). Mast cells are not only activated by specific allergens but also by various physical stimuli, e.g., rubbing, pressure, cold, heat which induce physical urticaria (Grabbe, 2001). These processes might be mediated through mechano- or thermosensitive TRP channels. Since ruthenium red inhibited the elevation of $[Ca^{2+}]_i$ and subsequent histamine release in RBL-2H3 cells induced by shear stress (Yang et al., 2009) and temperature increase (Stokes et al., 2004) or after irradiation with soft power lasers (Yang et al., 2007), a role of TRPV proteins was proposed in the above mast cell activating pathways. As Ruthenium Red inhibits several other channels in addition to TRPV channels, this concept requires validation by independent approaches.

Transcripts of *Trpv6* (formerly designated as Ca^{2+} transport protein 1, CaT1) have been detected in RBL-2H3 cells (Stokes et al., 2004). It had been proposed that TRPV6 comprises all or part of the CRAC pore (Yue et al., 2001) but later it was shown that TRPV6 expressed in HEK cells and CRAC in RBL-2H3 cells exhibit many differences in biophysical properties demonstrating that the pores of TRPV6 and CRAC are not identical (Voets et al., 2001). In this line, treatment of RBL-2H3 cells with antisense and siRNA probes directed against *Trpv6* transcripts, respectively, does not affect endogenous CRAC currents corroborating that TRPV6 is not a component of the native CRAC current in RBL mast cells. Nevertheless, expression of amino-terminal TRPV6 fragments, which are able to suppress currents through over-expressed TRPV6 channels, substantially suppressed activation of endogenous CRAC (Kahr et al., 2004).

TRPM CHANNELS

The members of the TRPM (melastatin) family are divided into four groups: TRPM1/3, TRPM2/8, TRPM4/5, and TRPM 6/7. For TRPM channels, heteromerization has only been reported for TRPM6 and TRPM7 so far (Chubanov et al., 2004, 2005; Li et al., 2006; Jiang, 2007). The Ca^{2+} permeability of channels formed by TRPM proteins ranges from monovalent selective (TRPM4 and TRPM5) to highly Ca^{2+} permeable (TRPM3α2, TRPM6, and TRPM7).

The founding member of this subfamily, *Trpm1* (initially termed melastatin), was initially identified as a gene that is downregulated in highly metastatic melanoma cells (Duncan et al., 1998). TRPM1 proteins reside in intracellular organelles and do not reach the plasma membrane upon heterologous expression, but form non-selective currents when expressed in SK-Mel22a melanoma cells (Oancea et al., 2009). TRPM1 is activated by the mGluR6 signaling cascade and thus is required for the depolarizing light response in ON bipolar cells (Morgans et al., 2009) and mutations in the *Trpm1* gene are associated with congenital stationary night blindness (CSNB) disease in humans (Li et al., 2009).

TRPM3 proteins are able to form constitutively active cation channels. Various splice variants are expressed from the *Trpm3* gene with TRPM3α1 being poorly permeable for divalent cations, whereas TRPM3α2-induced channels conduct Ca^{2+} and Mg^{2+}. The steroid hormone pregnenolone sulfate may act as endogenous ligand for TRPM3 (Wagner et al., 2008).

TRPM2 is activated by ADP-ribose (EC_{50}, 100 μM) and activated by H_2O_2 and under conditions of ROS production (Hara et al., 2002; Perraud et al., 2005), and deletion of *Trpm2* leads to decreased reactive oxygen species-induced chemokine production in monocytes (Yamamoto et al., 2008). Recently, Di et al. (2011) showed that deletion of *Trpm2* increases ROS production in phagocytes and introduced the concept that TRPM2-mediated cation entry and subsequent membrane depolarization functions

as an inhibitory feedback mechanism for ROS production in these cells.

TRPM4 and TRPM5 are (together with TRPM3α1) the only monovalent-selective ion channels of the TRP family (Launay et al., 2002; Hofmann et al., 2003). TRPM4, but not TRPM5, is inhibited by intracellular ATP, whereas TRPM5 is inhibited by intracellular acidic pH. Both are activated by an increase in Ca^{2+} levels in the cytosol, but the sensitivity to $[Ca^{2+}]_i$ as determined by different research groups varies greatly (Vennekens and Nilius, 2007). Studies in *Trpm4*$^{-/-}$ mice reveal a critical role for TRPM4 proteins in mast cell activation (see below). Moreover, these mice exhibited high blood pressure due to elevated release of catecholamines (Mathar et al., 2010). *Trpm5*$^{-/-}$ mice show abolished sweet, umami, and bitter taste reception (Zhang et al., 2003) and impaired glucose-induced insulin secretion (Colsoul et al., 2010).

TRPM6 and TRPM7 are unique among ion channels because they possess both ion channel and protein kinase activities. Channels formed by these proteins allow Mg^{2+} and Ca^{2+} entry into the cell and are inhibited by intracellular Mg^{2+} (0.3–1.0 mM; Nadler et al., 2001; Voets et al., 2004b). TRPM6 is primarily expressed in kidney and intestine, where it has been suggested to be responsible for epithelial Mg^{2+} reabsorption (Schlingmann et al., 2002). TRPM7 is ubiquitously expressed and deletion of the *Trpm7* gene leads to embryonic lethality (Jin et al., 2008; Weissgerber et al., 2008).

Like TRPM1, TRPM8 was originally identified in a screen of cancer-related genes (Tsavaler et al., 2001). It can be activated by cold (8–28˚C) and enhanced by cooling compounds such as menthol and icilin (McKemy et al., 2002) and *Trpm8*$^{-/-}$ mice show deficits in their ability to discriminate between cold and warm surfaces (Bautista et al., 2007). Temperature modulates the voltage dependence of the channel, and menthol and icilin mimick this effect (Voets et al., 2004a).

Expression of TRPM1, TRPM3, TRPM5, and TRPM6 has not been reported in mast cell models, while the other members of TRPM subfamily play important roles in mast cell functions. *Trpm2* transcripts were identified in human lung mast cells and cord blood derived mast cells in a microarray expression analysis (Bradding et al., 2003). In mouse BMMC, *Trpm4* transcripts were detected using Northern blot analysis and the 138 kDa TRPM4 proteins were identified in BMMCs of wild type but not of *Trpm4*$^{-/-}$ mice (Vennekens et al., 2007). After preabsorbtion of the same anti-TRPM4 antibody using microsomal membrane protein fractions from BMMCs of *TRPM4*$^{-/-}$ mice a specific staining of TRPM4 proteins in connective tissue mast cells in skin sections could be achieved. TRPM4 proteins, similarly like TRPM5 proteins, act as Ca^{2+}-activated non-selective cation channels and critically determine the driving force for Ca^{2+} influx into cells (Launay et al., 2004). It could be shown that TRPM4 channels depolarize the membrane following adenosine- and FcεRI-stimulation and, thereby, critically decrease the Ca^{2+} influx in BMMC's via CRAC channels. Accordingly, activated *Trpm4*$^{-/-}$ BMMCs release excessive histamine, leukotrienes and tumor necrosis factor (TNF), and *Trpm4*$^{-/-}$ animals display a more severe acute anaphylactic response in the skin compared to wild-type controls indicating that TRPM4 channel activation is an efficient mechanism for limiting antigen-induced mast cell activation (Vennekens et al., 2007). Additionally, antigen- and SCF-induced migration of BMMCs is largely diminished in the absence of TRPM4, and F-actin formation is reduced in DNP-HSA-stimulated BMMCs from *Trpm4*$^{-/-}$ mice (Shimizu et al., 2009). At this point it has to be mentioned that the increased $[Ca^{2+}]_i$ elevation observed upon FcεRI-stimulation in TRPM4-deficient mice led to increased release of TNF-α but not of IL-6. It is known, that release of both mast cell mediators is calcium-dependent, but an explanation for this difference may be that transcription and/or production of IL-6 may already be saturated under these conditions of FcεRI-stimulation and could not be further stimulated by the additional increase of $[Ca^{2+}]_i$ in TRPM4-deficient BMMCs.

Expression of TRPM7 has been found in human lung mast cells and the human cell line LAD-2 (Wykes et al., 2007) as well as in RBL-2H3 cells (Stokes et al., 2004) and Mg^{2+}-inhibited currents (MIC), which can be mediated by TRPM7 proteins, were identified in RBL-2H3 cells, HMC-1 cells, and human lung mast cells. Downregulation of TRPM7 expression in HMC-1 cells and human lung mast cells resulted in significant reduction of MIC currents and mast cell survival which could not be rescued by an increase in the extracellular Mg^{2+} concentration (Wykes et al., 2007). In addition, TRPM7 currents in RBL-2H3 cells might be involved in Ca^{2+} and Mg^{2+} entry during cell cycle regulation since it could be shown that MIC currents were strongly upregulated specifically in the G1 phase of the cell cycle to meet cellular demands for Ca^{2+} and/or Mg^{2+} fluxes during this stage of cell division (Tani et al., 2007). *Trpm7*-deficient mice as well as mice homozygous for an allele lacking the kinase domain of TRPM7 (*Trpm7*$^{\Delta kinase/\Delta kinase}$) die early during development (Jin et al., 2008; Weissgerber et al., 2008; Ryazanova et al., 2010), but *Trpm7*$^{+/\Delta kinase}$ are viable and MIC currents are significantly reduced in PMCs of these mice (Ryazanova et al., 2010). However, the consequences of MIC current reduction for mast cell activation have not been reported so far.

TRPM8 proteins, which form Ca^{2+}-conducting cation channels activated by menthol or cold, have also been proposed as mediators of mast cell activation, e.g., in the development of cold urticaria. However, Medic et al. (2011) found no deficits in mast cell activation using Ca^{2+} imaging, mast cell degranulation and development of passive anaphylaxis in *Trpm8*$^{-/-}$ mice, which makes a role of TRPM8 for mast cell activation in cold urticaria unlikely.

TRPA1 CHANNELS

TRPA1 is the only member of the TRPA (ankyrin) family characterized by the 14 amino-terminal ankyrin repeats (Story et al., 2003). Its expression in hair cells led to the hypothesis that it forms an auditory mechanotransduction channel (Corey et al., 2004), but this concept could not be supported sufficiently as, e.g., *Trpa1*$^{-/-}$ mice exhibit no overt vestibular deficits and auditory responses are completely normal (Bautista et al., 2006; Kwan et al., 2006). TRPA1 was reported to be activated by noxious cold but the thermosensitivity (Story et al., 2003; Corey et al., 2004) of TRPA1 is also debated. TRPA1 is activated by various chemicals (Bandell et al., 2004) including allyl isothiocyanate (the pungent compound in mustard oil), allicin (from garlic),

cinnamaldehyde (from cinnamon), menthol (from mint), tetrahydrocannabinol (from marijuana), nicotine (from tobacco), and bradykinin (see **Table 1**). Recently it was reported that protein kinase A/phospholipase C-mediated trafficking to the plasma membrane contributes to TRPA1 activation (Schmidt et al., 2009).

TRPA1 has been detected in mast cells so far, but in resting mast cells it was predominantly localized in intracellular vesicular structures and interacts with secretogranin III, a protein involved in secretory granule biogenesis in mast cells, implicating that TRPA1 might play an alternative role to the regulation of cation entry across the plasma membrane in mast cells compared to other cell types (Turner et al., 2007; Prasad et al., 2008).

TRPML AND TRPP CHANNELS

The TRPML (mucolipin) family contains three mammalian members: TRPML1, TRPML2, and TRPML3. The TRPML proteins show only low homology with the other TRP channels and are comparatively shorter. Heterologously expressed TRPML proteins can interact with each other (Venkatachalam et al., 2006; Curcio-Morelli et al., 2010). *Trpml1* was first identified by a positional cloning strategy as the gene mutated in patients suffering from Mucolipidosis type IV (MLIV; Bargal et al., 2000), TRPML3 was discovered as the channel mutated in varitint-waddler mice, characterized by a variegated coat color, vestibular defects, hyperactivity, and embryonic lethality (Xu et al., 2007). TRPML1 and TRPML2 are ubiquitously expressed and localize primarily to the lysosomal and late endosomal compartments (Manzoni et al., 2004; Puertollano and Kiselyov, 2009). Recently, Dong et al. developed a method allowing the measurement of ion currents directly in endolysosomes. This was achieved by treatment of the cells with Vacuolin-1, leading to an increase of diameter of the endolysosomes from 0.1–0.5 to 2–3 μM which makes them accessible for patch-clamp measurements. In this way, it could be shown that expressed TRPML1 proteins form constitutively active cation channels which, besides Na^+ and Ca^{2+}, can conduct several other cations, e.g., Mn^{2+}, Zn^{2+}, and Fe^{2+} out of the lumen of the organelles into the cytosol. The mechanism leading to activation of TRPML1 is still unclear (Dong et al., 2008, 2010).

The TRPP (polycystin) family comprises eight members, from which only PKD2 (TRPP2, PC2, polycystin-2), PKD2-L1 (TRPP3, polycystin-L), and PKD2-L2 (TRPP5, polycystin-L2) are shown to be channels. TRPP2 is reported to form a Ca^{2+}-permeable cation channel in the plasma membrane that can be activated by downstream of G protein-coupled receptor and/or receptor-tyrosine kinase at the cell (Ma et al., 2005), but was also shown to form a Ca^{2+} release channel in the ER (Wegierski et al., 2009). TRPP3 is reported to form Ca^{2+}-permeable non-selective cation channels with a large single channel conductance modulated by pH (Chen et al., 1999; Huang et al., 2006). TRPP5 is thought to form a Ca^{2+}-permeable non-selective cation channel (Guo et al., 2000). As indicated above, individual members of TRP subfamilies are able to interact. However, TRPP2 was the first example showing that heteromultimerization cannot only occur between members of the same subfamily since interaction of TRPP2 was reported with TRPC1 (Tsiokas et al., 1999; Bai et al., 2008; Kobori et al., 2009; Zhang et al., 2009) or with TRPV4 (Kottgen et al., 2008; Stewart et al., 2010). Recently, also another example of interaction of TRP proteins of different subfamilies, i.e., TRPV4 with TRPC1, has been described (Ma et al., 2010). It has to be mentioned that the nomenclature of TRPP proteins was changed recently (Clapham et al., 2012), and TRPP2 is now designated TRPP1, TRPP3 is now TRPP2, and TRPP5 is now TRPP3. Until now, members of the TRPML- or TRPP-channel subfamily were not identified in mast cells.

MECHANISMS: HOW TRP CHANNELS REGULATE CHANGES IN $[Ca^{2+}]_i$ AND MAST CELL ACTIVATION

Taken together, TRP channels were found to influence cellular Ca^{2+} signaling by several mechanisms. First, by conducting Ca^{2+} ions to various degrees TRP channels directly contribute to Ca^{2+} influx via the plasma membrane. TRP channels with high calcium selectivity are TRPV5 and TRPV6 (Vennekens et al., 2000), but also non-selective TRP channels composed of, e.g., TRPC1 (Suzuki et al., 2010) or TRPC5 (Ma et al., 2008) may contribute in this way. Second, by conducting Na^+ TRP channels mediate electrogenic effects through plasma membrane depolarization which may have opposite consequence in different cell types: in contrast to excitable cells, where TRP-mediated Na^+ entry and depolarization can enhance Ca^{2+} entry by gating of voltage-operated Ca^{2+} channels (Tsvilovskyy et al., 2009), they can do the opposite in non-excitable cells such as mast cells; here a significant part of stimulated Ca^{2+} entry enters the cell via inwardly rectifying CRAC channels, and TRP-mediated Na^+ entry and subsequent depolarization reduces the driving force for Ca^{2+} entry by shifting the membrane potential toward more positive potentials (Vennekens et al., 2007). A counteracting mechanism by a Ca^{2+} activated K^+ channel leading to membrane hyperpolarization and an increase of the driving force for Ca^{2+} entry following FcεRI-stimulation was shown for SK4 ($K_{Ca}3.1$) proteins (Shumilina et al., 2008) emphasizing the relevance of this regulatory principle for adjusting $[Ca^{2+}]_i$ and activation of mast cells. Third, TRP channels can be activated or inhibited by themselves by Ca^{2+} which further contributes to the complexity of regulation of $[Ca^{2+}]_i$. TRP channels that are activated by Ca^{2+} include TRPC1, TRPC4, TRPC5, TRPC6, TRPV4, TRPM2, TRPA1, TRPM4, and TRPM5. Also many TRP channels are modulated by Ca^{2+} via complex signaling cascades including Ca^{2+}/calmodulin binding, Ca^{2+}-dependent modulation of phospholipase C, and Ca^{2+}-dependent activation of PKC. Fourth, there is emerging evidence that several TRP channels are also located in the membrane of the sarco/endoplasmic reticulum (SR/ER), endosomes, lysosomes, or other intracellular vesicles where they can serve as Ca^{2+} release channels and conduct Ca^{2+} from the luminal stores into the cytoplasm which may affect specific mast cell functions; for instance, it was reported that TRPV1, TRPM8, and TRPP2 can form Ca^{2+}-conducting channels in the SR/ER, and TRPML1, TRPM2, and TRPV2 may function as Ca^{2+}-release channels in endo-/lysosomes (for more details see Dong et al., 2010; Gees et al., 2010). Finally, it has to be considered that many studies about Ca^{2+}-dependent mast cell functions analysed measurements of global cytoplasmic Ca^{2+} concentration rather than the nature of the Ca^{2+} signals, e.g., differences in the frequency of transient episodic Ca^{2+} elevations (Ca^{2+} spikes/oscillations) and subcellular localization and direction of Ca^{2+} signals such as Ca^{2+} waves which may be crucial for distinct mast cell functions. In this line, a threshold of Ca^{2+} elevation in the vicinity of store-operated

Ca^{2+} channels is apparently necessary to induce nuclear NFAT translocation as a parameter for activation of gene expression (Kar et al., 2011, 2012). In this case the signal triggered by this localized Ca^{2+} entry pathway might be achieved by spatially sequestered calmodulin molecules as mediators. Likewise, it would not be surprising if also a specific localization pattern of certain TRP channel proteins and their dynamic change during mast cell activation could be identified in the future as a mechanism that explains a specific pattern of mast cell activation such as activation of individual transcription factors or release of a defined set of mast cell mediators.

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Role of female sex hormones, estradiol and progesterone, in mast cell behavior

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Female sex hormones have long been suspected to have an effect on mast cell (MC) behavior. This assumption is based on the expression of hormone receptors in MCs as well as on the fact that many MC-related pathophysiological alterations have a different prevalence in females than in males. Further, serum IgE levels are much higher in allergic female mice compared to male mice. Ovariectomized rats developed less airway inflammation compared to sham controls. Following estrogen replacement ovariectomized rats re-established airway inflammation levels' found in intact females. In humans, a much higher asthma prevalence was found in women at reproductive age as compared to men. Serum levels of estradiol and progesterone have been directly correlated with the clinical and functional features of asthma. Around 30–40% of women who have asthma experienced worsening of their symptoms during the perimenstrual phase, the so-called perimenstrual asthma. Postmenopausal women receiving hormone replacement therapy have an increased risk of new onset of asthma. Beside, estrus cycle dependent changes on female sex hormones are related to changes on MC number in mouse uterine tissue and estradiol and progesterone were shown to induce uterine MC maturation and degranulation. We will discuss here the currently available information concerning the role of these female sex hormones on MC behavior.

Keywords: degranulation, estradiol, mast cells, progesterone, uterus

INTRODUCTION

Mast cells (MCs) belong to the innate-compartment of the immune system and are widely known for their role in allergic reactions via their binding to IgE receptor (Alvarez-Errico et al., 2009). MCs are a common cellular component of both connective and mucosal tissues (Kitamura and Ito, 2005). Beside this, MCs contain a wide range of biologically active molecules, including biogenic amines, heparin or heparan sulfate proteoglycans, neutral proteases, and neuropeptides. In addition, upon stimulation, they also produce and eject a large number of factors (Wilhelm et al., 2000). Taking these characteristics together, it is clear that even a small number of such potent unicellular glands have a significant effect on different physiological processes.

In addition to the very well known and described mechanism of MC activation and posterior degranulation throughout IgE receptor, several other alternative but not redundant mechanisms of MC activation have been described (Mousli et al., 1994; Bradding, 2005; Kim et al., 2008). Among others, female sex hormones, estradiol and progesterone, have been proposed to activate MC (Chancey et al., 2005; Vasiadi et al., 2006; Narita et al., 2007; Zaitsu et al., 2007; Jensen et al., 2010; Jing et al., 2011; Walter et al., 2011). We will discuss in this review the current bibliography evidences about the effect of female sex hormones on MC functionality.

MAST CELLS EXPRESS ESTRADIOL AND PROGESTERONE RECEPTORS AND FURTHER RESPOND TO THESE HORMONES

Female sex steroid hormones act primarily via their receptors: estrogen via estrogen receptor ERα or ERβ, progesterone via progesterone receptor PR-A or PR-B (Carey et al., 2007). Steroid receptors are best described as nuclear receptors acting as transcription factors on gene expression. However, in the past decade abundant evidences accumulated showing addition binding sides localized at the plasma membrane (Levin, 2011), whose activation is more often involved in the rapid effects of steroids occurring within seconds to minutes (Watson et al., 1999; Watson and Gametchu, 2003). In this regard, it has been shown that classical ERα at the membrane but not in the nucleus mediates 17β-estradiol (E2)-induced rapid signaling to kinase activation (Levin, 2011). Similarly, extra-nuclear PR induces activation of ERK/MAPK kinases, which lead to cell surviving as well as cells migration (Levin, 2011). We and other authors have demonstrated the expression of, estradiol and progesterone receptors in human, mouse, and rat MCs (Theoharides et al., 1993; Chancey et al., 2005; Pang et al., 1995; Narita et al., 2007; Zaitsu et al., 2007; Jensen et al., 2010; Jing et al., 2011). Zaitsu et al. (2007) have shown mRNA expression of ERα but not ERβ in human and mouse MCs. Alongside the authors have also shown that E2 rapidly stimulated MC degranulation which could be blocked by tamoxifen, a tissue specific ER antagonist, clearly indicating that

estradiol-induced MC degranulation throughout one of its receptors. Bone marrow-derived MCs (BMMCs) isolated from ERα knockout animals did not degranulate in response to E2 treatment confirming that the E2 effect on MCs is more likely mediated by the ERα (Zaitsu et al., 2007). Due to the rapid onset of E2 effect on MC activation the authors concluded that E2 in this context does not function through the classical (genomic) mechanisms, which require enhanced mRNA and protein synthesis over 2 h or longer period and proposed that the effect is mediated by a membrane-associated (non-genomic) form of ER (Zaitsu et al., 2007). We were additionally able to show that the human mast cell line (HMC-1) treated *in vitro* with physiological concentration of E2 and P4 significantly increased the synthesis of β-tryptase, which is a serine proteinase abundantly produced by MCs, and is a marker of MC maturation. Beside, E2 and P4 treatment induced degranulation of HMC-1 *in vitro* (Jensen et al., 2010).

Supporting the idea of female sex hormones having an effect on MC function, Kirmaz et al. (2004) have demonstrated that allergen skin prick tests (SPT), a very sensitive and specific tests to detect allergic sensitization in atopic patients, is altered in women upon hormonal changes during the menstrual cycle.

In addition to female sex hormone receptor expression, MCs have been also shown to express androgen receptor (Chen et al., 2010). However, testosterone treatment had no effect on MC degranulation (Chen et al., 2010).

INFLUENCE OF ESTRADIOL AND PROGESTERONE ON MC FUNCTION: DO THESE HORMONES PLAY A ROLE IN MC-RELATED DISEASES?

The idea that female sex hormones, E2 and P4, may affect MC functionality and therefore have an influence on the symptoms of MC-associated disorders has long been suggested. Asthma and other allergic diseases of the airway are up to three times more common in women than in men during the early to middle adulthood and remains so through the reproductive years (De Marco et al., 2002; Mannino et al., 2002; Schatz and Camargo, 2003). A number of clinical and epidemiological studies suggested that female sex hormones are accountable for these differences. Beside this, postmenopausal women taken hormone replacement therapy had higher risk of new onset of asthma (Barr et al., 2004). Furthermore, 30–40% of women who had asthma, experience a worsening of their symptoms during the perimenstrual phase of the menstrual cycle (perimenstrual asthma) being the time point when E2 and P4 concentrations are changing rapidly (Vrieze et al., 2003). In this context, it is of great importance to mention that the prevalence and morbidity of asthma and other allergic diseases have increased dramatically during the last 30 years, particularly in developing countries (Burr et al., 2006). Narita et al. (2007) have nicely demonstrated that this may be related to the increase of low concentrations of environmental like-estrogen compounds. These estrogen-like compounds, called xenoestrogens, are present in the environmental pollutants mainly in water and food. They are able not only to activate MCs but enhance MC degranulation upon allergen cross-linking of IgE which may explain the above described increment of allergic diseases in the last years in developing countries (Narita et al., 2007).

In an animal model of allergic disease, the role of female sex hormone was tested. Female mice have reportedly an increased susceptibility to allergic airway disease in compared with male mice (reviewed in Carey et al., 2007). Levels of IgE are much higher in allergic female mice compared to their syngeneic male (Corteling and Trifilieff, 2004). Female rats that underwent ovariectomization developed less airway inflammation compared with sham controls animals (Ligeiro de Oliveira et al., 2004). However, estrogen replacement in the ovariectomized animals re-established airway inflammation levels of intact females (Ligeiro de Oliveira et al., 2004). Treatment of intact female rats with the selective estrogen receptor antagonist tamoxifen also reduced the development of allergic airway disease (Ligeiro de Oliveira et al., 2004). Thus, the direct effect of these hormones on disease development is hereby demonstrated.

Beyond the well-documented effects of estradiol and progesterone on MC function in MC-associated diseases, these hormones were further implicated in controlling different MC process under physiological conditions. For instance, estradiol was showed to be a potent inducer of ovarian MC degranulation, which seems to be a necessary factor during the process of oocyte ovulation (Jaiswal and Krishna, 1996; Tamura and Kogo, 1999).

MC NUMBER, MATURATION, AND DEGRANULATION IN THE UTERUS ARE UNDER THE CONTROL OF FEMALE SEX HORMONES

The presence of MCs in the uterus has been already described in many species including human (Drudy et al., 1991), mouse (Padilla et al., 1990), rat (Aydin et al., 1998), hamster (Harvey, 1964) as well as goat (Karaca et al., 2008). Besides, the number of MCs in the uterus was shown to fluctuate during estrous cycle suggesting an influence of female sex hormones on MC recruitment to the uterus (Aydin et al., 1998). Ovariectomized mice, in which estradiol and progesterone are almost absent, have less number of uterine MCs compared to control, non-ovariectomized animals (Jensen et al., 2010). Hormonal replacement, estradiol alone or in combination with progesterone, restored the number of uterine MCs after ovariectomization, which was comparable to the levels observed in control mice (Jensen et al., 2010). Hormonal replacement additionally induced an augmentation in the levels of MC-related proteases expression in the uterus as well as boosted MC degranulation (Jensen et al., 2010). This is of particular importance because upon degranulation, MCs release several molecules (histamine, proteases, metalloproteinases, pro-angiogenic factors), all very well known to account for the process of embryo implantation.

CONCLUSION

Mast cells, the so-called unicellular glands, once solely known as effectors cells of the innate immune system only activated by IgE cross-linking to the IgE receptor upon allergen stimulation are now known to be much more plastic and susceptible to be activated by several factors including female sex hormones, estradiol and progesterone. Strong data in the last years reinforced the idea that these hormones are crucial component of MC behavior not

only in physiological conditions but also in several MC pathological situations. Deciphering the mechanisms by which female sex hormones activate MCs and under which conditions these happens, alongside with explanation why female sex hormones have these effects is of crucial interest for a better understanding of the physiology of these cells.

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TLR signaling in mast cells: common and unique features

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In addition to the well known role of mast cells in immunity to multi-cellular parasites and in the pathogenesis of allergy and asthma, the importance of mast cells in the immune defense against bacteria and viruses is increasingly being recognized. Their location in the skin, gut, and airways puts mast cells in an ideal location to encounter and respond to pathogens, and in order to perform this function, these cells express a variety of pattern recognition receptors, including Toll-like receptors (TLRs). Mast cells respond to TLR ligands by secreting cytokines, chemokines, and lipid mediators, and some studies have found that TLR ligands can also cause degranulation, although this finding is contentious. In addition, stimulation via TLR ligands can synergize with signaling via the FcεRI, potentially enhancing the response of the cells to antigen *in vivo*. A great deal is now known about TLR signaling pathways. Some features of these pathways are cell type-specific, however, and work is under way to fully elucidate the TLR signaling cascades in the mast cell. Already, some interesting differences have been identified. This review aims to address what is known about the responses of mast cells to TLR ligands and the signaling pathways involved. Given the location of mast cells at sites exposed to the environment, the response of these cells to TLR ligands must be carefully regulated. The known mechanisms behind this regulation are also reviewed here.

Keywords: cytokine, innate, mast cells, review, signaling, TLRs

INTRODUCTION

Host cells utilize a variety of germline encoded receptors termed pathogen recognition receptors (PRRs), including the Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD) proteins, to recognize pathogens. These receptors allow the innate immune system to identify invading bacteria by their expression of pathogen-associated molecular patterns (PAMPs; Akira et al., 2006). Signaling via these receptors guides the immune system to mount the correct response to an invading pathogen, or to a harmless commensal, by a process which is not well understood (Blander and Sander, 2012).

Mast cells have traditionally been known for their roles in allergy and immunity to multi-cellular parasites (Metcalfe et al., 1997) but increasingly the crucial roles that they play in immune defense against bacteria and viruses are being recognized (Marshall, 2004; Abraham and St John, 2010). Mast cells are able to recognize pathogens via their expression of PRRs and by binding to antibodies with the FcRs (Abraham and St John, 2010). This review will focus on TLR expression, function and signaling, since the TLRs are the best studied PRR on mast cells.

Abbreviations: AC, adenylate cyclase; BMMC, bone marrow-derived mast cell; Btk, Bruton's tyrosine kinase; CBMC, cord blood-derived mast cell; cysLT, cysteinyl leukotriene; FSDMC, fetal skin-derived mast cells; IFN, interferon; IGF-1, insulin-like growth factor-1; IHC, immunohistochemistry; LBP, LPS-binding protein; MD-2, myeloid differentiation-2; n.d., not determined; P3C, tripalmitoyl Cys-Ser-(Lys)$_4$; PAMPs, pathogen-associated molecular patterns; PBDMC, peripheral blood-derived mast cell; PCDMC, peritoneal cell-derived mast cell; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C.

The TLRs are a family of receptors which recognize a wide variety of PAMPs, as summarized in **Table 1**. Furthermore, it is increasingly being recognized that certain endogenous molecules which are expressed during tissue damage or disease are also TLRs agonists (Kawai and Akira, 2010). There are 10 human TLRs, TLR1–TLR10, while 13 are found in the murine genome, TLR1–TLR9 and TLR11–TLR13 (Lee et al., 2012). The receptors largely function as homodimers, with the exception of TLR2 which forms heterodimers with both TLR1 and TLR6 (Akira et al., 2006; Lee et al., 2012). The TLR2 homodimers and heterodimers are located on the cell surface, as are TLR4 and TLR5, while TLR3 and TLR7–TLR9 are endosomally located, allowing them to recognize intracellular nucleic acids (Lee et al., 2012). Among other ligands, TLR4 recognizes LPS, TLR5 binds flagellin, and TLR2 heterodimers recognize various lipopeptides (Akira et al., 2006; Lee et al., 2012; **Table 1**).

Much work has been carried out to determine the signaling pathways triggered by the TLR receptors and the consequences of their ligation (Lu et al., 2008; Akira, 2009; Kawai and Akira, 2010). This review aims to address the ability of mast cells to respond to TLR ligands and to examine what is known about TLR signaling and its regulation in mast cells. In addition, cross-talk between the TLR signaling pathways and that of FcεRI has been identified (Avila and Gonzalez-Espinosa, 2011), and the mechanisms and consequences of this will be discussed.

TLR EXPRESSION ON MAST CELLS

Several studies have been undertaken on murine and human mast cells isolated *ex vivo*, or differentiated from stem cells, as

Table 1 | The main TLR ligands (adapted from Akira et al., 2006; Lee et al., 2012).

TLR	Physiological ligands	Synthetic ligands
TLR1–2	Triacylated lipopeptides (bacteria and mycobacteria)	Pam_3CSK_4
TLR2	Peptidoglycan (gram positive bacteria), phospholipomannan (*Candida albicans*), tGPI-mucins (*Trypanosoma*), haemagglutinin (measles virus), porins (*Neisseria*), lipoarabinomannan (mycobacteria), glucuronoxylomannan (*Cryptococcus neoformans*), HMGB1 (host)	
TLR2–6	Diacylated lipopeptides (*Mycoplasma*), LTA (Group B *Streptococcus*), zymosan (*Saccharomyces cerevisiae*)	FSL1, MALP-2, Pam_2CSK_4
TLR3	dsRNA (viruses)	PolyI:C
TLR4	LPS (Gram-negative bacteria), VSV glycoprotein G, RSV fusion protein, MMTV envelope protein, mannan (*Candida albicans*), glucuronoxylomannan (*Cryptococcus neoformans*), glycosylinositolphospholipids (*Trypanosoma*), HSP60, HSP70, fibrinogen, HMGB1 (all host proteins), nickel	
TLR5	Flagellin (Flagellated bacteria)	
TLR7	ssRNA (RNA viruses)	Imidazoquinoline compounds: imiquimod, resiquimod, loxoribine, R848
TLR8	ssRNA(RNA viruses)	Resiquimod
TLR9	CpG-DNA (bacteria and mycobacteria), DNA (viruses), haemozoin (*Plasmodium*)	CpG-A, CpG-B and CpG-C ODNs

well as on mast cell lines to establish which TLRs are expressed. The findings of these studies are summarized in **Table 2**. The TLRs appear to be widely expressed by murine mast cells, with expression of TLR1–4 and 6–9 identified at least at the mRNA level (McCurdy et al., 2001; Supajatura et al., 2001; Masuda et al., 2002; Ikeda and Funaba, 2003; Matsushima et al., 2004; Li et al., 2009; Mrabet-Dahbi et al., 2009). Expression of TLR5 has not been demonstrated on murine mast cells (McCurdy et al., 2001; Supajatura et al., 2001; Ikeda and Funaba, 2003; Matsushima et al., 2004).

Expression of TLR1–10 with the exception of TLR8 has been identified on human mast cells, although some studies were unable to identify TLR1, 4, 6, or 9 (McCurdy et al., 2003; Okumura et al., 2003; Varadaradjalou et al., 2003; Kulka et al., 2004; Kulka and Metcalfe, 2006; Yoshioka et al., 2007). TLR expression on the mast cell lines LAD2, HMC-1, and MC-9 has been assessed with varied results in different studies (McCurdy et al., 2001; Masuda et al., 2002; Kulka et al., 2004; Kulka and Metcalfe, 2006; Kubo et al., 2007; Yoshioka et al., 2007). It should be noted that several receptors have only been detected at the mRNA level and that further work will be required to demonstrate protein expression.

The expression of TLR2 by mast cells has been studied in more detail and it has been suggested that bone marrow-derived mast cell (BMMC) do not express the whole TLR2 protein but rather a truncated protein lacking the intracellular signaling domain (Mrabet-Dahbi et al., 2009). Despite this, a range of studies have determined that mast cells are able to respond to TLR2 ligands, as discussed below, and this may be due to the fact that the truncated TLR2 is still able to form heterodimers (Mrabet-Dahbi et al., 2009).

MAST CELL RESPONSES TO TLR STIMULATION

MAST CELL RESPONSES TO CELL SURFACE TLRs

Acting via TLR4, LPS caused IL-6, IL-13, and TNFα secretion from murine BMMC (McCurdy et al., 2001; Supajatura et al., 2001) and a later study found secretion of IL-5 and IL-10 upon LPS stimulation via TLR4 (Masuda et al., 2002). In addition to these cytokines, LPS stimulation of murine BMMC and fetal skin-derived mast cells (FSDMC) also caused the secretion of the chemokines CCL3/MIP-1α and CXCL2/MIP-2 (Matsushima et al., 2004; **Figure 1**).

Differences between the cytokines produced upon TLR4 and TLR2 stimulation have been observed: LPS caused murine BMMC to secrete TNFα, IL-6, IL-13, and IL-1β via TLR4; while peptidoglycan (PGN) causes the secretion of TNFα and IL-6, in addition to the Th2 cytokines, IL-4, IL-5, and IL-13 via TLR2 (Supajatura et al., 2002; **Figure 1**). In rat peritoneal mast cells, both PGN and LPS resulted in cysteinyl leukotriene production, but the response to PGN was greater (Wierzbicki and Brzezinska-Blaszczyk, 2009). Taken together, these findings suggest that mast cells release a wider variety of mediators in response to PGN than LPS. This appears not to be the case in macrophages, where stimulation with LPS or PGN has been shown to lead to an up-regulation of similar mRNAs (Wang et al., 2000).

Murine peritoneal cell-derived mast cells (PCDMC) responded more potently to TLR agonists than BMMC and it is suggested that the PCDMC are more mature than BMMC, and this increased maturity underlines their increased ability to respond to TLR stimulation (Mrabet-Dahbi et al., 2009). LTA and MALP-2 treatment of PCDMC resulted in IL-1, IL-6, IL-17, GM-CSF, IL-10, TNFα, and IFNγ production, while LPS caused only IL-6, GM-CSF, IL-10, and TNFα secretion from PCDMC (Mrabet-Dahbi et al., 2009).

Table 2 | TLR expression by mast cells.

	Murine	Human	Cell line
TLR1	+ BMMC mRNA[1]	− lung mRNA[2]	+ HMC-1 mRNA and protein[3]
	+ FSDMC mRNA[1]	− skin mRNA[2]	− LAD2 mRNA and protein[3,5]
		+ PBDMC mRNA and protein[3]	+ LAD2 protein[5]
		+ CBDMC mRNA[4]	
TLR2	+ BMMC mRNA[1,6−9]	+ lung mRNA[2]	− HMC-1 mRNA and protein[3,11]
	+ FSDMC mRNA[1]	+ lung protein[5]	+ LAD2 mRNA and protein[3,5,11]
	+ BMMC protein[9]	+ skin mRNA[2]	+ MC-9 mRNA[7]
	+ PCDMC protein[9]	+ PBDMC mRNA and protein[3]	
		+ CBDMC mRNA[4]	
		+ CBDMC mRNA and protein[10]	
		+ in polyps by IHC[4]	
TLR3	+ BMMC mRNA[1]	+ lung mRNA[2]	+ LAD1 protein[2]
	+ FSDMC mRNA[1]	+ skin mRNA[2]	+ HMC-1 mRNA and protein[3]
		+ PBDMC mRNA and protein[3]	+ LAD2 mRNA and protein[3]
		+ Bone marrow[3]	
TLR4	+ BMMC mRNA[1,6−9,12]	+ lung mRNA[2]	+ MC-9 mRNA[7,12]
	+ BMMC protein[1,9,13]	+ skin mRNA[2]	− HMC-1 mRNA and protein[3]
	+ FSDMC mRNA and protein[1]	+ PBDMC mRNA and protein[3,14]	+ HMC-1 mRNA and protein[11]
	+ peritoneal protein[1]	+ CBDMC mRNA and protein[10]	+LAD2 mRNA and protein[3,5,11]
	+ PCDMC protein[9]	− CBDMC mRNA[4]	
TLR5	− BMMC mRNA[1,6−8]	+ lung mRNA[2]	+ HMC-1 mRNA and protein[3]
	− FSDMC mRNA[1]	+ skin mRNA[2]	+ LAD2 mRNA and protein[3,5]
		+ PBDMC mRNA and protein[3]	− MC-9 mRNA[7]
TLR6	+ BMMC mRNA[1,6−9]	− lung mRNA[2]	+ HMC-1 mRNA and protein[3]
	+ FSDMC mRNA[1]	− skin mRNA[2]	+ LAD2 mRNA and protein[3]
		+ PBDMC mRNA and protein[3]	+ MC-9 mRNA[7]
		+ CBDMC mRNA[4]	
TLR7	+ BMMC mRNA[1]	+ lung mRNA[2]	+ HMC-1 mRNA and protein[3]
	+ FSDMC mRNA[1]	+ skin mRNA[2]	+ LAD2 mRNA and protein[3]
		+ PBDMC mRNA and protein[3]	
TLR8	+ BMMC mRNA[8]	− lung mRNA[2]	+ HMC-1 mRNA and protein[3]
		− skin mRNA[2]− PBDMC mRNA and protein[3]	− LAD2 mRNA and protein[3]
TLR9	− BMMC mRNA[1]	+ lung mRNA[2]	+ HMC-1 mRNA and protein[3]
	+ FSDMC mRNA[1]	− skin mRNA[2]	+ LAD2 mRNA and protein[3,5]
	+ peritoneal protein[1]	+ PBDMC mRNA and protein[3]	
TLR10	No murine homolog	+ lung mRNA[2]	None tested
		+ skin mRNA[2]	

TLR expression on mast cells is summarized. The references are indicated by superscript numbers: [1] *Matsushima et al., 2004;* [2] *Kulka and Metcalfe, 2006;* [3] *Kulka et al., 2004;* [4] *McCurdy et al., 2003;* [5] *Yoshioka et al., 2007;* [6] *Ikeda and Funaba, 2003;* [7] *McCurdy et al., 2001;* [8] *Supajatura et al., 2001;* [9] *Mrabet-Dahbi et al., 2009;* [10] *Varadaradjalou et al., 2003;* [11] *Kubo et al., 2007;* [12] *Masuda et al., 2002.*

Of these three agonists, only LTA-induced PGD_2 production in PCDMC (Mrabet-Dahbi et al., 2009; **Figure 1**).

In human cord blood-derived mast cells (CBMC), stimulation with zymosan or PGN caused GM-CSF, IL-1β, LTB_4, and LTC_4 production (Olynych et al., 2006). Another study also identified differences between the mediators released upon different TLR ligand stimulation: PGN, zymosan, and Pam_3Cys caused GM-CSF and IL-1β secretion whereas LPS did not; and PGN and zymosan

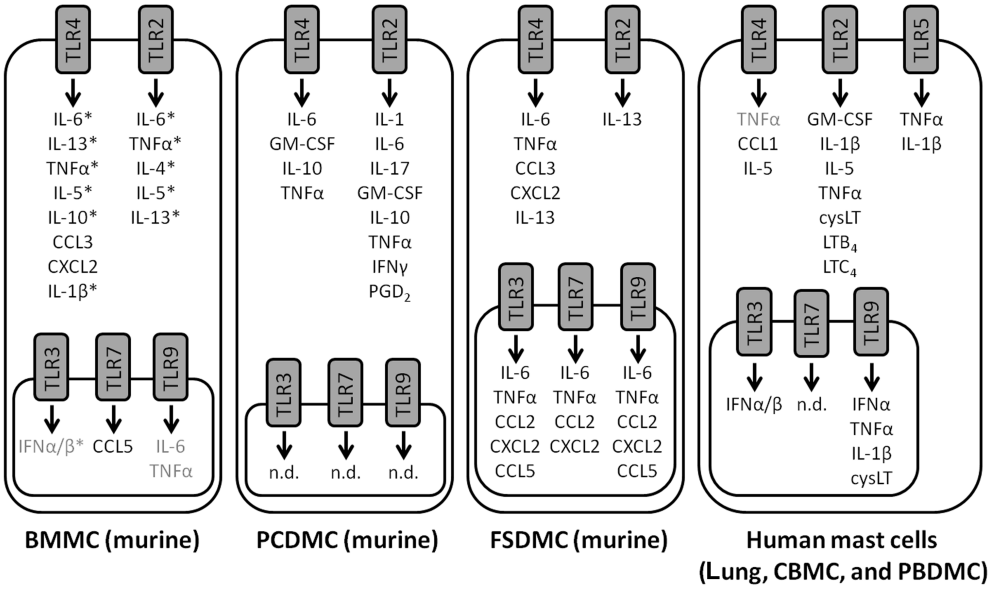

FIGURE 1 | Mast cell secretory responses to TLR ligation. A diagram showing the molecules secreted by mast cells upon TLR ligation. The cytokines, chemokines, and lipid mediators released upon TLR ligand stimulation are summarized for murine BMMC, PCDMC, FSDMC, and human mast cells. Where there is discrepancy in the literature, molecules are shown in grey. *indicates instances where it has been demonstrated with the use of TLR-deficient cells or blocking antibodies that the ligand is acting via the indicated receptor.

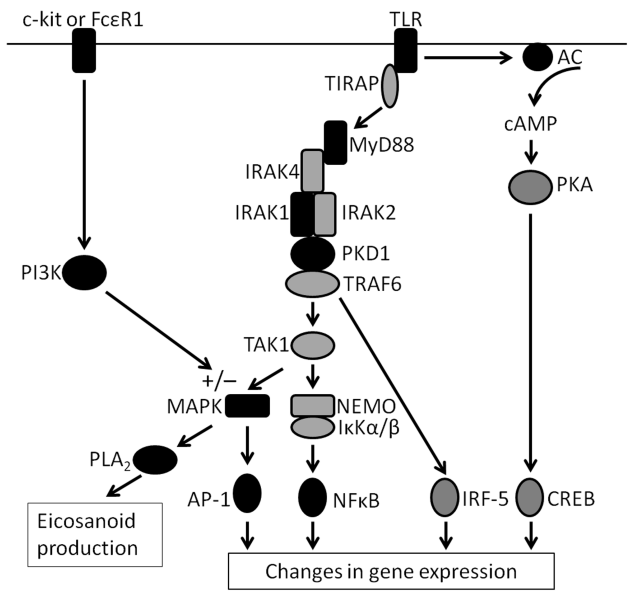

FIGURE 2 | TLR signaling in the mast cell. A scheme illustrating the signaling pathways triggered by TLR ligation in the mast cell. Where there is evidence in the literature for the involvement of a particular protein, the protein is filled in black. Proposed molecules are in grey (adapted from Akira et al., 2006; Lu et al., 2008; Park et al., 2009). The activation of PI3K via c-kit or FcεRI stimulation is shown, with its inhibitory and activating effects on MAPK activation.

treatment led to the production of LTC_4 unlike Pam_3Cys treatment (McCurdy et al., 2003). Human mast cells cultured from $CD34^+$ progenitors isolated from blood (PBDMC) stimulated with LPS produced significant amounts of TNFα, whereas PGN induced IL-1β, GM-CSF, IL-5, and cysteinyl leukotriene in addition to TNFα (Kulka et al., 2004; **Figure 1**). Therefore, as has been shown in murine mast cells, stimulation via TLR2 results in a greater range of mediator production than stimulation via TLR4.

Pre-treatment of mast cells with cytokines has been shown to enhance the response of the cells to TLR ligands (Okumura et al., 2003; Varadaradjalou et al., 2003). In one study, LPS only induced TNFα production after the CBMC had been incubated with IL-4, whereas even untreated cells were able to respond to PGN (Varadaradjalou et al., 2003). It is not clear why the cells in this study were unable to respond to the TLR4 agonist without TNFα pre-treatment (Varadaradjalou et al., 2003), unlike human PBDMC (Kulka et al., 2004). Human lung mast cells and PBDMC responded to LPS by secreting TNFα but this response, and TLR4 expression, was increased by pre-treatment with IFNγ (Okumura et al., 2003). This group also noticed CCL1 and IL-5 production in LPS-treated lung mast cells but not PBDMC, and gene array analysis showed that LPS caused the up-regulation of a variety of genes including a protease, several cytokines, chemokines, receptors, and STAT5a (Okumura et al., 2003).

Peptidoglycan has been demonstrated to induce migration of peritoneal rat mast cells after a short treatment with TNFα (Brzezinska-Blaszczyk and Rdzany, 2007), and in a later publication, LPS and PGN both caused migration of the cells after treatment with IL-6 or CCL5/RANTES, respectively (Wierzbicki and Brzezinska-Blaszczyk, 2009). The mechanism behind these effects is as yet unknown, but it has been suggested that IL-6 and CCL5/RANTES may modulate TLR expression on the mast cells (Wierzbicki and Brzezinska-Blaszczyk, 2009). The ability of TLR agonists to cause mast cell migration *in vivo* would allow PAMPs or

endogenous TLR ligands produced upon tissue damage to recruit mast cells to sites of infection or inflammation.

TLR5 expression has been more readily detected on human than murine mast cells (see **Table 1**) and human PBDMC respond to flagellin (a TLR5 ligand) by secreting IL-1β and TNFα, demonstrating that the receptor is functional on these cells (Kulka et al., 2004; **Figure 1**). To our knowledge, flagellin has not been shown to cause cytokine secretion from murine mast cells, in agreement with the lack of detectable expression of TLR5 on the cells (McCurdy et al., 2001; Supajatura et al., 2001; Ikeda and Funaba, 2003; Matsushima et al., 2004).

MAST CELL RESPONSES TO INTRACELLULAR TLRs

Double-stranded RNA molecules, such as polyI:C, are used as a synthetic mimic of viral RNA (see **Table 1**) and cause IFNα and β secretion from human PBDMC and murine BMMC (Kulka et al., 2004). This response was partially blocked with anti-TLR3 antibodies and in TLR3$^{-/-}$ BMMC, suggesting that the receptor is involved in the detection of the RNA (Kulka et al., 2004). A different group found that murine FSDMC responded far more robustly to polyI:C than BMMC, secreting IL-6, TNFα, CCL2/MIP-1α, CXCL2/MIP-2, and CCL5/RANTES which was in agreement with the greater TLR3 expression by FSDMC than BMMC (Matsushima et al., 2004). In a recent study using BMMC, no IL-6, TNFα or IFNα/β production was observed upon polyI:C treatment (Keck et al., 2011; **Figure 1**). The ability of BMMC to respond to TLR3 stimulation is, therefore, somewhat controversial, although mast cells from other sources clearly do respond (Kulka et al., 2004; Matsushima et al., 2004). These finding may reflect the more immature phenotype of BMMC.

Murine BMMC have been found to respond to bacterial but not mammalian DNA, and to synthetic oligonucleotides containing an unmethylated cytosine followed by a guanosine (CpG motif), by secreting IL-6 and TNFα (Zhu and Marshall, 2001). MC/9 cells were also found to respond to CpG-containing oligonucleotides by secreting IL-6 and TNFα, and the response of BMMC was greater when greater numbers of CpG sequences were included in the oligonucleotides (Zhu and Marshall, 2001). These treatments were not found to induce mast cell degranulation or the secretion of GM-CSF, IL-4, IL-12, or IFNγ (Zhu and Marshall, 2001). A later study comparing BMMC and FSDMC found that TLR9 was expressed by FSDMC but not BMMC, and demonstrated TNFα, IL-6, CCL2/MIP-1α, CXCL2/MIP-2, and CCL5/RANTES secretion by FSDMC but not BMMC treated with CpG-containing oligonucleotides (Matsushima et al., 2004; **Figure 1**).

The TLR7 agonist, R848, caused secretion of IL-6 and TNFα and also the chemokines CCL2/MIP-1α and CXCL2/MIP-2 from FSDMC but not BMMC, and TLR7 expression was far higher in FSDMC (Matsushima et al., 2004). In spite of this, R848 stimulation of BMMC did lead to some CCL5/RANTES production (Matsushima et al., 2004; **Figure 1**), therefore murine mast cells appear to respond to TLR7 agonists, in agreement with their expression of the receptor (see **Table 2**).

A study using human PBDMC found that CpG-containing oligonucleotides stimulated cells to produce IFNα, IL-1β, TNFα, and cysteinyl leukotriene (Kulka et al., 2004). CpG-containing oligonucleotides activate TLR9 therefore these data suggest that in addition to expressing TLR9 (see **Table 2**), both human and murine mast cells are able to respond to TLR9 ligands by secreting cytokines and lipid mediators. The sensitivity of mast cells to TLR7 and TLR9 agonists would presumably assist in the immune defense against bacteria, viruses, and *Plasmodium* (see **Table 1**).

Fetal skin-derived mast cells express higher levels of TLR3, TLR7, and TLR9 and respond more potently to agonists of these receptors than BMMC (Matsushima et al., 2004), in a similar manner to the greater response of FSDMC than BMMC to TLR2 and 4 agonists (Mrabet-Dahbi et al., 2009). These results are likely a reflection of the immaturity of BMMC and suggest that responses to some TLRs are better studied in mast cell models other than BMMC (Matsushima et al., 2004; Mrabet-Dahbi et al., 2009).

Work performed in other immune cells has demonstrated that TLR3 and 9 signaling requires endosomal acidification and maturation, presumably because these receptors are intracellularly located (Ahmad-Nejad et al., 2002; Matsumoto et al., 2003; Akira, 2009). Similarly in FSDMC, the cytokine secretion induced by polyI:C (TLR3 ligand), R848 (TLR7 ligand), and CpG (TLR9 ligand) was inhibited by an inhibitor of endosomal maturation (Matsushima et al., 2004). In contrast, the mast cell response to LPS was unaffected by the treatment (Matsushima et al., 2004), in agreement with similar studies in a macrophage cell line (Ahmad-Nejad et al., 2002), presumably because TLR4 binds LPS at the cell surface.

THE EFFECT OF TLR LIGATION ON MAST CELL DEGRANULATION AND PHENOTYPE

In addition to these findings that stimulation of mast cells with TLR agonists leads to cytokine and chemokine production and mast cell migration, some data suggest that mast cell degranulation can be induced by TLR2 ligands. Stimulation of BMMC with PGN resulted in mast cell degranulation whereas stimulation via TLR4 did not (Supajatura et al., 2002). Similar results were obtained in human mast cells (Varadaradjalou et al., 2003). Stimulation of CBMC with PGN led to histamine release in addition to cytokine release, whereas LPS stimulation caused only cytokine secretion (Varadaradjalou et al., 2003). In another study using human CBMC, the degranulation induced by PGN was not found to be statistically significant, while zymosan and Pam_3Cys induced significant degranulation (McCurdy et al., 2003). *In vivo*, i.d. injection of PGN but not LPS caused a mast cell-dependent increase in vascular permeability, indicating that the TLR2 ligand was inducing mast cell degranulation (Supajatura et al., 2002).

Other groups have been unable to demonstrate degranulation after stimulation of mast cells with TLR ligands, however. Neither LPS nor PGN induced degranulation of BMMC (Ikeda and Funaba, 2003) or rat peritoneal mast cells (Wierzbicki and Brzezinska-Blaszczyk, 2009), and studying BMMC and FSDMC, Matsushima et al. (2004) did not detect degranulation in response to LPS, PGN, polyI:C, R848, or CpG, suggesting that signaling via TLR2–4, 7, and 9 does not cause mast cell degranulation. In agreement with these findings, TLR1/TLR2, TLR2/TLR6, and TLR4 agonists were not observed to cause degranulation of MC/9

cells or BMMC in another study (Qiao et al., 2006). Degranulation was not observed in BMMC or the more mature PCDMC in response to the TLR2 ligands MALP-2, LTA, or PGN (Mrabet-Dahbi et al., 2009). When these agonists were given i.p., no drop in body temperature was observed, suggesting that TLR2 activation does not lead to degranulation of mast cells *in vivo* (Mrabet-Dahbi et al., 2009).

It is difficult to reconcile the differences in the findings of these various studies. It may be that differences in the cell culture or isolation methods, or differences in the agonist preparations used could explain the discrepancies.

In other settings, TLR ligands have inhibited mast cell degranulation. Stimulation of LAD1 cells with dsRNA analogues resulted in decreased adhesion of the cells to fibronectin and vitronectin via TLR3, which led to a decrease in the degranulation observed when cells were allowed to adhere to these proteins (Kulka and Metcalfe, 2006). LTA and PGN acted over 24–48 h to downregulate surface levels of FcεRI expression on LAD2 cells and human lung mast cells, which resulted in a decreased degranulation after antigen exposure (Yoshioka et al., 2007). The effect was only partially mediated by TLR2 and was not observed with TLR4, 5, or 9 agonists (Yoshioka et al., 2007).

These findings suggest that TLR signaling may affect the phenotype of mast cells, for example by downregulating FcεRI expression (Yoshioka et al., 2007). Human mast cells cultured *in vitro* in the presence of LPS or PGN had altered protease composition and cytokine production profiles (Kirshenbaum et al., 2008). In another study, LPS has been shown to cause an increase in TLR4 expression in LAD2 cells, such that increased levels of TNFα were produced after a second stimulation with LPS (Kubo et al., 2007). This is in contrast to work performed on BMMC where classical endotoxin-tolerance was observed and cells were unresponsive to a second LPS challenge (Sly et al., 2004; Saturnino et al., 2010).

In conclusion, the ability of TLR2 agonists to cause mast cell degranulation is controversial and further work is required to clarify the situation. It seems that TLR stimulation affects the mast cell phenotype modulating the levels of receptors and proteases. Exposure of mast cells to TLR agonists *in vivo*, therefore, may control their ability to respond to other stimuli and the type of response they are able to mount.

TLR SIGNALING IN THE MAST CELL

LACK OF TRIF-DEPENDENT PATHWAY IN TLR4 SIGNALING IN THE MAST CELL

The prototypical TLR4 ligand is LPS which is bound by the secreted protein, LPS-binding protein (LBP) and transferred to the TLR4 signaling complex by cell secreted or membrane bound CD14 (Lee et al., 2012). TLR4 acts in a complex with MD-2 (Lee et al., 2012) and this has also been shown to be the case in mast cells (Ushio et al., 2004). It is now recognized that CD14 is only required for the cell to recognize rough LPS but not smooth LPS (which contains full-length *O*-chains; Jiang et al., 2005; Huber et al., 2006; Lee et al., 2012).

Conventionally, TLR4 signaling proceeds via two signaling pathways: the MyD88-dependent and the TRIF-dependent (MyD88-independent) pathways (Lu et al., 2008; Akira, 2009; Kawai and Akira, 2010). Activation of the MyD88-dependent pathway leads to the production of pro-inflammatory cytokines via activation of AP-1, IRF-5, and NF-κB. This pathway requires the adaptor protein, TIRAP, to mediate the interaction between TLR4 and MyD88. The adaptor TRAM is required for TLR4 to activate the TRIF pathway which leads to the activation of IRF-3 and, therefore, interferon-β (IFNβ) production. This pathway also causes a delayed NF-κB activation which contributes to the production of pro-inflammatory cytokines (Lu et al., 2008; Akira, 2009; Kawai and Akira, 2010).

In BMMC, it appears that TLR4 signaling proceeds only via the MyD88-dependent pathway and that the TRIF-dependent pathway is not used (Keck et al., 2011). LPS stimulation of mast cells does not lead to IFN production (Dietrich et al., 2010; Keck et al., 2011) and TRIF deficiency does not affect the BMMC cytokine secretion induced by LPS (Keck et al., 2011). In addition, LPS-induced NF-κB activation is entirely dependent on MyD88 (**Figure 2**; Keck et al., 2011).

BMMC were observed to express reduced levels of TRAM, the adaptor protein that links TRIF to the TLR4 receptor complex (Keck et al., 2011). This reduction in TRAM may prevent TLR4, and therefore LPS, from activating the TRIF pathway (Keck et al., 2011). It does not appear to fully account for the defect, however, as although TRAM over-expressing BMMC produced increased IL-6 upon LPS stimulation, IFN production was still not detected (Keck et al., 2011).

TLR4 signaling via the MyD88-dependent pathway occurs from the cell membrane, whereas TRIF-mediated signaling is believed to occur in early endosomes after internalization of the TLR4 complex (Kagan et al., 2008). TLR4 is not internalized after stimulation on mast cells (Dietrich et al., 2010; Keck et al., 2011), and while in macrophages LPS is transported inside the cell, this is not the case in mast cells (Dietrich et al., 2010). It seems likely that this lack of internalization may explain why the TRIF-dependent pathway does not occur in mast cells. CD14 is required for the internalization of TLR4 (Zanoni et al., 2011; Lee et al., 2012) and although BMMC express CD14 mRNA (Ikeda and Funaba, 2003), they do not express detectable levels of CD14 on the cell surface, and CD14 must be provided in serum for the response to LPS (McCurdy et al., 2001; Varadaradjalou et al., 2003). Therefore, this lack of CD14 may explain why TLR4 is not efficiently internalized in mast cells and, therefore, why the TRIF pathway is not activated by LPS (Keck et al., 2011).

The lack of CD14 on the cell surface may not fully explain the inability of LPS to stimulate the TRIF pathway in mast cells, however, since IFNα/β production (albeit reduced) is observed upon LPS stimulation of $CD14^{-/-}$ macrophages suggesting that CD14-independent TRIF activation is possible (Keck et al., 2011). It is not clear whether this is due to a limited degree of CD14-independent internalization of TLR4 or if the TRIF pathway is activated from the plasma membrane in this condition (Keck et al., 2011).

The inability of LPS to stimulate the TRIF-dependent pathway is not unique to mast cells. Neutrophils stimulated with LPS similarly produce no IFN (Tamassia et al., 2007). It has been suggested that the lack of TRIF signaling in response to LPS may be a protective mechanism to prevent excessive activation of mast cells by

the commensal bacteria routinely encountered by the cells due to their location at sites close to the interface with the environment (Keck et al., 2011). The same may be true for other cell types and it is not known how many other cells respond in this way to TLR4 stimulation (Tamassia et al., 2007).

In addition to its key role in TLR4 signaling, CD14 is known to be involved in the responses of other TLRs to various ligands, although the molecular details are not fully understood (Lee et al., 2012). Therefore, the lack of surface CD14 may affect the response of mast cells to TLR2 and TLR5 ligands. Since CD14 mRNA has been detected in mast cells (Ikeda and Funaba, 2003) it is possible that the protein is available intracellularly and may be involved with TLR3 and TLR7–9 signaling. Further studies will be required to determine CD14 intracellular expression in mast cells and what function this protein may play in TLR signaling.

Further extending these findings that LPS treatment does not cause IFNα/β secretion, Keck et al. (2011) demonstrated that neither infection with an adenoviral vector nor B-DNA transfection, both of which stimulate macrophages to produce IFNβ, caused IFN production in mast cells. Gene array analysis of human cells found that whilst a group of interferon response genes were upregulated by LPS in monocytes, this upregulation did not occur in mast cells (Okumura et al., 2003). The lack of type I IFN production upon LPS stimulation of human mast cells was confirmed by quantitative PCR, suggesting that the IFN response to LPS is lacking in human mast cells as it is in murine mast cells (Okumura et al., 2003; Keck et al., 2011).

Mast cells are not entirely defective in IFN production, however. Infection with vesicular stomatitis virus caused IFN production (Keck et al., 2011), and polyI:C treated human and murine mast cells secrete IFNα and β (Kulka et al., 2004) although this finding was not reproduced in a later study (Keck et al., 2011). It seems that the ability of mast cells to mount a potent IFN response is tightly regulated.

ACTIVATION OF IRAKs

The family of interleukin-1 receptor-associated kinases (IRAKs) are involved in the downstream signaling of TLRs (Akira, 2009; Kawai and Akira, 2010). Very little is known about their roles in TLR signaling in mast cells. In MC-9 cells, LPS and P3C were shown to activate IRAK1 in an *in vitro* kinase assay, suggesting that this kinase is important in signaling from TLR4 and TLR2/TLR1 (**Figure 2**; Qiao et al., 2006). Future studies are needed to address the roles played by IRAK1 other members of this family in TLR signal transduction in the mast cell.

MAPK ACTIVATION BY TLR LIGANDS IN MAST CELLS

The MAPKs are known to play a key role in TLR signaling in immune cells (Akira, 2009; Kawai and Akira, 2010) and their role in TLR signaling in the mast cell has been addressed in several studies. The involvement of p38, Erk, and Jnk in mast cell TLR signaling has been demonstrated, although there are some discrepancies between studies. There seems to be more support for a role of p38 and Jnk in the TLR signaling pathways in the mast cell than for Erk, although some studies have identified Erk as an important player.

TLR4, TLR2/1, and TLR2/6 signaling activated p38 MAPK in BMMC in a comparable manner to stimulation through FcεRI (Zorn et al., 2009). Furthermore, an inhibitor of p38 reduced the IL-6 produced upon stimulation with LPS, Pam_3CSK_4, or FSL-1, suggesting that this kinase plays a role in TLR2 and 4 signaling (Zorn et al., 2009). Interestingly, inhibition of p38 phosphorylation caused a reduction in the secretion of IL-13 and IL-10 from BMMC upon LPS stimulation but did not reduce the mRNAs of these cytokines, suggesting that p38 regulates the production of these cytokines post-transcriptionally (Masuda et al., 2002).

In addition to p38 phosphorylation, Masuda et al. (2002) detected Jnk1/2 and p38 activation after LPS stimulation of MC-9 cells and BMMC which was similar to that induced by FcεRI signaling, together with a weaker Erk1/2 phosphorylation. In contrast, in a separate study, Jnk1/2 phosphorylation was not detected in BMMC after LPS stimulation (Supajatura et al., 2001). It has been suggested that this discrepancy may be explained by the sensitivity of the assays used, since Jnk1/2 activation was demonstrated in an *in vitro* kinase assay in the later study while Jnk-phosphorylation was undetectable by western blot (Masuda et al., 2002). A more recent study on BMMC revealed phosphorylation of p38, Jnk1/2, and Erk1/2 after LPS stimulation but no Erk5 phosphorylation which was induced by stimulation of FcεRI (Li et al., 2009).

Inhibition of Jnk with curcumin reduced the amount of IL-10 and IL-13 but not IL-5 produced by BMMC and MC/9 cells upon LPS stimulation, and similar results were obtained after overexpression of a dominant negative Jnk in MC/9 cells (Masuda et al., 2002). Production of the anti-microbial peptide, CRAMP upon LPS stimulation of BMMC was not dependent on the MAPK p38, Jnk1/2, or Erk (Li et al., 2009).

The IFNα production induced upon polyI:C stimulation of human PBDMC was inhibited by pharmacological inhibitors of Jnk and p38, suggesting that these pathways are also involved in TLR3 signaling in mast cells (Kulka et al., 2004). In support of these findings, Jnk and p38 phosphorylation was observed after stimulation of human PBDMC with polyI:C (Kulka et al., 2004).

Treatment of LAD2 cells with LPS, LTA, PGN, flagellin, or CpG-containing oligonucleotides resulted in phosphorylation of Erk (Yoshioka et al., 2007). In MC/9 cells, however, neither LPS nor the TLR2/TLR1 ligand, P3C was observed to cause Erk phosphorylation while both ligands induced detectable Jnk and p38 phosphorylation (Qiao et al., 2006). Specific inhibitors of all three MAPKs reduced the TNFα production induced by the ligands, suggesting that Erk does play a role in the TLR signaling in MC/9 cells, even though Erk phosphorylation was not detected (Qiao et al., 2006). In a separate study, however, pharmacological inhibition of Erk phosphorylation had no effect on the IL-5, -10, or -13 secretion induced by LPS in either MC/9 or BMMC (Masuda et al., 2002).

Pam_3CSK_4 induced Erk-phosphorylation in BMMC that was dependent on TLR2 and MyD88, and an inhibitor of MEK, the MAPK upstream of Erk, reduced the LTC_4 and PGD_2 production induced by Pam_3CSK_4, confirming the importance of Erk in this signaling pathway in mast cells (Kikawada et al., 2007). Sustained Erk phosphorylation was not observed in mast cells deficient for the group V secretory PLA_2, and as a result the amount

of leukotriene and prostaglandin produced upon stimulation of these cells with Pam_3CSK_4 was reduced (**Figure 2**; Kikawada et al., 2007). The crucial role of the PLA_2 on signaling seems to be specific to the TLR2 pathway as the deficient mast cells responded as wild-type cells to SCF and to stimulation through FcεRI (Kikawada et al., 2007).

In conclusion, it seems that p38, Jnk, and Erk are all involved in TLR signaling in the mast cell, and that their relative predominance depends on the cells and stimuli type and concentration used, as well as the particular cytokine of interest.

REQUIREMENT FOR ADENYLATE CYCLASE

It has been demonstrated in epithelial cells that the IL-6 production observed upon TLR4 stimulation is dependent upon the secondary messenger cAMP activating the transcription factor CREB (Song et al., 2007). Similarly in mast cells, inhibition of adenylate cyclase (AC) in CBMC reduced the IL-6 production in response to PGN and Pam_3CSK_4, but had no effect on the IL-1β produced (Haidl et al., 2011; **Figure 2**). This finding suggests that cAMP is important in the mast cells response to TLR2 stimulation, although interestingly, it may be redundant in the production of IL-1β (Haidl et al., 2011).

CALCIUM SIGNALING AND PROTEIN KINASE ACTIVATION IN TLR SIGNALING IN THE MAST CELL

Calcium is not thought to be involved in TLR signaling pathways (Lu et al., 2008; Akira, 2009; Kawai and Akira, 2010), but is an important secondary messenger in the FcεRI signaling pathway in the mast cell which leads to degranulation (Gilfillan and Tkaczyk, 2006). Since some studies have demonstrated that TLR ligands cause mast cell degranulation (Supajatura et al., 2002; McCurdy et al., 2003; Varadaradjalou et al., 2003), while others have been unable to reproduce these findings (Ikeda and Funaba, 2003; Matsushima et al., 2004; Qiao et al., 2006; Mrabet-Dahbi et al., 2009; Wierzbicki and Brzezinska-Blaszczyk, 2009), it is perhaps not surprising that similar discrepancies exist in the literature describing the ability of TLR agonists to cause calcium release in mast cells.

In MC-9 cells and BMMC, LPS, PGN, MALP-2, and P3C were unable to induce calcium signaling (Qiao et al., 2006). In contrast, it has been shown in BMMC that PGN causes calcium mobilization which was dependent on TLR2, but LPS did not have this effect (Supajatura et al., 2002). As discussed, the discrepancies between these two studies are difficult to reconcile.

Protein kinase C (PKC) α and β appear to have no role in LPS signaling in the mast cell, since BMMC deficient in either kinase responded as well as wild-type cells to LPS, and an inhibitor of PKCs had only minimal effects on the response (Zorn et al., 2009).

Similarly, PKCs do not appear to play an important role in TLR2 signaling in the mast cell, since an inhibitor of PKC did not reduce the levels of CCL2/MCP-1 produced upon Pam_3CKS_4 stimulation of BMMC (Murphy et al., 2007). PKD1, however, was shown to be activated in BMMC upon treatment with the TLR2 agonist, Pam_3CKS_4 (**Figure 2**; Murphy et al., 2007). The phosphorylation of PKD1 was dependent on MyD88 and reduced levels of CCL2/MCP-1 mRNA and protein were produced by cells when a PKD inhibitor was added, suggesting that the kinase is important in the response of the cells to TLR2 ligands (Murphy et al., 2007). A more recent study in macrophages also identified a crucial role for PKD1 in Myd88-dependent TLR signal transduction (Park et al., 2009). Further work will be required to understand the roles of PKD1 and other protein kinases in TLR signaling in the mast cell.

ROLE OF BTK IN MAST CELL TLR SIGNALING

Bruton's tyrosine kinase (Btk) interacts with several TLR receptors and components of the TLR signaling pathway including IRAK1 and TIRAP, and the kinase is activated by LPS in THP-1 cells (Jefferies et al., 2003). The role this kinase plays in TLR signaling is controversial. In one study, mononuclear cells from patients with mutations in Btk showed an impaired TNFα response to LPS, demonstrating that this kinase is required for TLR4 signaling (Horwood et al., 2003). Whereas, another study on monocytes from patients deficient in Btk found no such defect in TLR4 signaling (Perez de Diego et al., 2006). Murine $Btk^{-/-}$ macrophages produced reduced levels of IL-10 in response to several TLR agonists than wild-type cells, which resulted in an increase in the amount of IL-6 produced (Schmidt et al., 2006).

Btk is important in signaling through the FcεRI (Gilfillan and Tkaczyk, 2006), demonstrating that the tyrosine kinase is expressed and functional in mast cells. Btk does not appear to play a vital role in TLR signaling in the cells, however, since the response to TLR4, TLR2/TLR1, and TLR2/TLR6 ligands was either unaffected or enhanced in BTK-deficient mast cells (Zorn et al., 2009). Phosphorylation of p38 upon LPS stimulation was unaffected by Btk deficiency in BMMC (Zorn et al., 2009). These data suggest that the kinase may have an inhibitory role in TLR signaling in the mast cell, in contrast to that which has been described in monocytes and macrophages (Horwood et al., 2003; Schmidt et al., 2006; Zorn et al., 2009).

ACTIVATION OF TRANSCRIPTION FACTORS

Roles for several transcription factors have been demonstrated in TLR activation, including AP-1-binding proteins (such as c-jun and c-fos) and NF-κB (Akira, 2009; Kawai and Akira, 2010) and some of these have been implicated in TLR signaling in the mast cell.

In BMMC, LPS and PGN caused phosphorylation of IκB-α at Ser32 (Supajatura et al., 2002) which would lead to NF-κB activation. In an earlier publication, it was demonstrated that IκB-α phosphorylation after LPS stimulation only occurred in C3H/HeN BMMC and not in BMMC derived from the C3H/HeN TLR4-mutated strain, demonstrating that this activation was induced via TLR4 (Supajatura et al., 2001), and this was supported by the lack of IκB-α phosphorylation after LPS stimulation of $TLR4^{-/-}$ BMMC (Supajatura et al., 2002). Similarly, IκB-α phosphorylation was not observed in $TLR2^{-/-}$ BMMC after PGN stimulation (Supajatura et al., 2002).

A more recent study detected limited IκBα degradation upon LPS stimulation and greater levels of IκBβ degradation, particularly at time points of over an hour, confirming that NFκB signaling occurs upon TLR4 signaling in mast cells (Li et al., 2009). Inhibition of this pathway reduced the levels of transcription of

an anti-microbial peptide, demonstrating the importance of this pathway in the response (Li et al., 2009).

NF-κB signaling is also implicated in TLR signaling in human mast cells. IκB phosphorylation was detected in human PBDMC after polyI:C stimulation, and the IFNα induced upon polyI:C treatment of the cells was inhibited with a chemical inhibitor of NF-κB (Kulka et al., 2004). In human CBMC, PGN and Pam_3CSK_4 induced IL-6 and IL-1β production was inhibited by an inhibitor of IκK-2, suggesting that the NF-κB pathway is also important in TLR2 signaling in human mast cells (Haidl et al., 2011). In agreement with these findings that NF-κB is activated upon TLR2 and TLR4 stimulation of mast cells, NF-κB-binding activity was detected in nuclear extracts of MC/9 cells after stimulation with LPS and P3C (Qiao et al., 2006).

Taken together, these studies clearly define an important role for NF-κB in TLR2 and TLR4 signal transduction in human and murine mast cells (**Figure 2**). P3C and LPS treatment of MC/9 cells resulted in phosphorylation of ATF-2 and, to a lesser extent, c-Jun implying that these two transcription factors are involved in the signal transduction pathways of TLR2 and 4 in mast cells (**Figure 2**; Qiao et al., 2006). In the same study, c-fos activity was not induced by either ligand, nor was STAT 3, 5, or 6 activation detected (Qiao et al., 2006). Further work will be required to determine whether these transcription factors are activated in human and murine mast cells in addition to this cell line.

As discussed above, in stark contrast to the situation in macrophages, LPS stimulation of mast cells does not lead to IFN production (Dietrich et al., 2010; Keck et al., 2011). This is reflected in the activation of the transcription factor IRF-3 (Keck et al., 2011). In macrophages, LPS treatment causes IRF-3 phosphorylation which is not observed in BMMC, even when soluble CD14 is added to the media. When LPS is administered i.p., IRF-3 phosphorylation was observed by flow cytometry in macrophages but not mast cells, demonstrating that this difference between the two cell types also exists *in vivo* (Keck et al., 2011).

EFFECT OF PI3K SIGNALING

Phosphoinositide 3-kinase (PI3K) is composed of a p110 catalytic subunit and a p85 regulatory subunit and its action produces lipid mediators which act as secondary messengers and activate downstream kinases. The PI3K pathway is a regulator of TLR signaling which can have either positive or negative effects on signaling depending on cell type and stimulus, as reviewed by Hazeki et al. (2007).

Since PI3K activation leads to phosphorylation of the kinase AKT, AKT phosphorylation can be used as a readout for PI3K activation. AKT phosphorylation was not detected after LPS or P3C activation of MC/9 cells, suggesting that these TLR4 and TLR2 ligands do not activate the PI3K pathway in these cells (Qiao et al., 2006).

Inhibition of the PI3K pathway with two pharmacological inhibitors reduced the amount of TNFα, IL-6, and IL-1β produced by BMMC upon LPS stimulation (Sly et al., 2004). In a more recent study, however, whilst Wortmannin reduced the amount of TNFα and IL-6 produced while the IL-1β production was increased, suggesting that the pathway differentially regulates cytokine production in mast cells (Hochdorfer et al., 2011). These disparate findings regarding the role of PI3K signaling in IL-1β production are difficult to reconcile, particularly since both studies used BMMC and similar concentrations of Wortmannin (Sly et al., 2004; Hochdorfer et al., 2011). Co-treatment of cells with LPS and known PI3K stimulating factors such as IGF-1 caused an increase in the amount of TNFα produced, but inhibited the production of IL-1β in murine BMMC and PCDMC (Hochdorfer et al., 2011; **Figure 2**).

The differential effects of PI3K activation on TNFα and IL-1β is intriguing, and a similar result was obtained in human monocytes, in that the inhibition of PI3K differentially affected the production of two cytokines (Martin et al., 2003). When monocytes were stimulated with LPS in the presence of PI3K inhibitors, the amount of IL-12 produced was increased whilst the amount of IL-10 produced was inhibited (Martin et al., 2003). The mechanism behind the disparity appears to be that inhibition of PI3K led to suppression of Erk1/2 activation, and Erk has been previously demonstrated to cause the production of IL-10 and suppress IL-12 production in RAW264.7 cells (Yi et al., 2002). Perhaps a similar mechanism is at work in mast cells, and may explain the opposing effect that PI3K activation has on TNFα and IL-1β production.

Activation of the SCF receptor, c-kit, potently induces PI3K signaling in mast cells, and there are several mutations of the c-kit receptor which are associated with human disease that result in constitutive c-kit activation (Robyn and Metcalfe, 2006). This raised the interesting possibility that mast cells in patients with particular c-kit mutations may respond differently to stimulation with LPS. Indeed, the L138.8A mast cell line which contains such a c-kit activating mutation did not produce IL-1β upon LPS stimulation unless PI3K signaling was chemically inhibited (Hochdorfer et al., 2011). It is interesting to speculate that mast cell responses to TLR agonists *in vivo* may be modulated by other stimuli that the cell encounters that activate the PI3K signaling pathway.

ACTIVATION OF INHIBITORY PATHWAYS

Several pathways that inhibit TLR signaling have been identified which presumably act to prevent over-reaction of cells to TLR ligands which could result in immune-mediated pathology (Kawai and Akira, 2010). The presence of some of these pathways has been investigated in mast cells, and several have been shown to be functional in the cells.

In macrophages, TIRAP becomes phosphorylated and degraded by SOCS1 after TLR2 and TLR4 activation, which consequently prevents further signaling via the MyD88-dependent pathway (Mansell et al., 2006). This inhibitory pathway does not occur in mast cells, however, and the levels of TIRAP remain unchanged after stimulation (Zorn et al., 2009). In mast cells, LPS activation leads to a reduction in the levels of mRNA of SOCS1 and CISH (a SOCS family member), whereas in macrophages it results in an increase in the levels of SOCS1, SOCS3, and CISH, which are thought to be responsible for the degradation of TIRAP (Mansell et al., 2006; Zorn et al., 2009).

The SH2-containing inositol phosphatase (SHIP) inhibits the NF-κB pathway during FcεRI stimulation of mast cells

(Kalesnikoff et al., 2002) and has been shown to be upregulated in both mast cells and macrophages after LPS stimulation (Sly et al., 2004). The ability of SHIP to negatively regulate TLR4 signaling is illustrated by the demonstration that injection of a sub-lethal concentration of LPS was lethal in SHIP deficient animals (Sly et al., 2004). SHIP inhibits signaling through the PI3K pathway (Huber et al., 1998) and since inhibition of PI3K inhibits the LPS-induced cytokine production in mast cells (Sly et al., 2004; Hochdorfer et al., 2011), it seems logical that SHIP would inhibit TLR4 signaling in these cells. Indeed, SHIP-mediated negative feedback has been shown to be important in the phenomenon of endotoxin-tolerance in both mast cells and macrophages, since endotoxin-tolerance could not be induced in $SHIP^{-/-}$ mast cells or macrophages (Sly et al., 2004). The SHIP expression in LPS stimulated mast cells and macrophages is caused by autocrine TGFβ, implying that this regulatory cytokine is important in inhibiting the response to LPS in both cell types (Sly et al., 2004).

As previously discussed, inhibition of the PI3K pathway in mast cells does not inhibit all cytokine production stimulated by LPS. Rather, the production of IL-1β was enhanced when the pathway was inhibited (Hochdorfer et al., 2011). In support of this, whilst LPS stimulation of $SHIP^{-/-}$ mast cells resulted in greater TNFα production, the amount of IL-1β secreted was reduced (Hochdorfer et al., 2011).

DAP12 is a transmembrane protein which has been shown to inhibit the response of macrophages to TLR agonists (Hamerman et al., 2005). $DAP12^{-/-}$ BMMC, however responded to TLR4, TLR2/TLR1, and TLR2/TLR6 agonists in a comparable manner to wild-type cells, suggesting that the signaling pathways in mast cells are independent of DAP12 (Smrz et al., 2010).

TANK is a negative regulator of TLR signaling (Kawagoe et al., 2009) and has been identified in gene array analysis as being upregulated in LPS stimulated mast cells (Okumura et al., 2003), raising the possibility that it may be involved in a negative feedback loop. Further work will be required to determine whether this protein indeed inhibits TLR signaling, and to establish what other pathways are important in the regulation of TLR-mediated mast cell activation.

Other as yet unidentified mechanisms may be in place to limit the response of the mast cell to LPS, or perhaps, since this does not result in IFN production (Dietrich et al., 2010; Keck et al., 2011), regulation of the mast cell LPS response is less crucial than that of macrophages. Indeed, it has been suggested that the lack of TRIF signaling in response to LPS may be a protective mechanism to prevent excessive activation of mast cells by the commensal bacteria routinely encountered by the cells due to their location at sites close to the interface with the environment (Keck et al., 2011).

RECEPTOR CROSS-TALK BETWEEN TLRs AND OTHER RECEPTORS ON MAST CELLS

Dectin-1 is a PPR which is known to interact with several TLRs, and behaves as a co-receptor for TLR2 (Gantner et al., 2003; Reid et al., 2009). Dectin-1 is believed to be primarily expressed on myeloid cells, and has been shown to be expressed on human mast cells (Olynych et al., 2006). Inhibition of dectin-1 reduced the LTC_4 produced upon CBMC stimulation with zymosan but not PGN, and did not inhibit the production of GM-CSF or IL-1β (Olynych et al., 2006). These data suggest that the PPR is involved in mast cell recognition of zymosan, presumably in conjunction with TLR2, but not of PGN, and that the receptor is required for cell signaling to induce the production of lipid mediators but not cytokines (Olynych et al., 2006). In support of this, pharmacological inhibition of the tyrosine kinase Syk, which is activated by dectin-1, inhibited the production of LTC_4 induced by zymosan and to a lesser extent by PGN. Syk is therefore important in the downstream signaling from TLR2 in mast cells (Olynych et al., 2006).

Stimulation of mast cells via TLRs results in cytokine and chemokine production in a similar way to that observed for other cells (Akira et al., 2006; Kawai and Akira, 2010). Mast cells are unique in that they express the FcεRI in addition to TLRs, and so there is the potential for cross-talk between these two cell stimulatory pathways. Several studies have addressed the impact of TLR signaling on stimulation of mast cells via the FcεRI and vice versa.

Mast cells sensitized with IgE respond more robustly to LPS stimulation (Medina-Tamayo et al., 2011). This enhanced sensitivity is not due to an increase in the expression of TLR4, CD14, or MD-2, rather the cells appear to be "pre-activated" by binding IgE and show higher basal levels of NF-κB activation (Medina-Tamayo et al., 2011). This finding adds to earlier demonstrations that IgE binding to the FcεRI activates mast cells to some extent (Kawakami and Kitaura, 2005). In addition, the anti-apoptotic effect of monomeric IgE on mast cells is synergistically enhanced by the addition of LPS, signaling via TLR4, although LPS alone had no effect on apoptosis (Jayawardana et al., 2008).

In addition to these effects, the FcεRI signaling pathway shares many features with TLR signaling, for example both pathways utilize MyD88 (Gilfillan and Tkaczyk, 2006; Akira, 2009; Kawai and Akira, 2010), therefore there is potential for cross-talk between the two pathways (Avila and Gonzalez-Espinosa, 2011).

The cytokine production of BMMC and MC/9 cells upon stimulation via the FcεRI receptor is synergistically enhanced in the presence of the TLR4 agonist, LPS, and the TLR2/TLR1 agonist P3C, and to a lesser extent by that of MALP-2 and PGN (both TLR2/TLR6 agonists; Qiao et al., 2006). In contrast, the degranulation response is unaffected (Qiao et al., 2006).

Stimulation of BMMC with the TLR2 ligands MALP-2 and Pam_3CSK_4 synergizes with stimulation through FcεRI to enhance IL-6 production (Fehrenbach et al., 2007). MALP-2 had no effect on FcεRI-induced degranulation whereas Pam_3CSK_4 inhibited antigen-induced degranulation, although this was found to be due to a direct interaction between the model antigen and the lipid itself, rather than any cross-talk between signaling pathways (Fehrenbach et al., 2007).

Signaling through FcεRI induces PI3K activation in mast cells (Yano et al., 1993; Gilfillan and Tkaczyk, 2006) and, as previously discussed, inhibition of the PI3K pathway during LPS stimulation results in an inhibition of IL-6 and TNFα (although the effect on IL-1β production is contentious), suggesting that this pathway acts to increase responses to LPS (Sly et al., 2004; Hochdorfer et al., 2011; **Figure 2**). In agreement with this, stimulation of BMMC via

FcεRI enhances the IL-6 and TNF induced by LPS, whilst inhibiting the IL-1β production (Hochdorfer et al., 2011).

In MC/9 cells, synergy has also been observed between TLR4 and FcεRI induced Jnk and p38, but not Erk phosphorylation (Masuda et al., 2002; Qiao et al., 2006). The use of selective MAPK inhibitors, however, did suggest a role for Erk signaling in the synergy between the two signaling pathways (Qiao et al., 2006). In contrast, Smrz et al. (2010) found no evidence of synergy between FcεRI and TLR signaling in the activation of p38, Erk, or Jnk. The activation of the transcription factors, ATF-2, c-Jun, and c-Fos upon stimulation via FcεRI was increased in the presence of either TLR2 or TLR4 stimulation (Qiao et al., 2006).

Synergy was not observed for the calcium response induced by antigen, indeed, P3C was shown to inhibit the calcium release induced by mast cell activation via the FcεRI pathway by an unknown mechanism (Qiao et al., 2006). Similarly, the IRAK1 activation caused by the TLR ligands was slightly inhibited by antigen stimulation (Qiao et al., 2006).

Synergy between TLR and FcεRI signaling pathways therefore has been demonstrated (Qiao et al., 2006; **Figure 2**), and results in enhanced cytokine secretion but not degranulation (Qiao et al., 2006; Fehrenbach et al., 2007; Hochdorfer et al., 2011). Much of this work has been performed in murine mast cells and it would be interesting to investigate the phenomenon in the human context. It has been proposed that the increased response to stimulation via FcεRI in the presence of TLR2 and 4 ligands may contribute to the worsening of allergic symptoms which can occur in the presence of pathogens (Qiao et al., 2006).

CONCLUSION

Recent research identifies important roles for mast cells in the immune defense against bacteria and pathogens (Marshall, 2004; Abraham and St John, 2010) and given their locations at sites of microbial entry into the host (Metcalfe et al., 1997) the ability of the cells to recognize invading pathogens must be crucial. A variety of PRRs are responsible for initial recognition of pathogens (Akira et al., 2006) and of these, the TLRs are the best studied in mast cells.

Mast cells have been shown to express the majority of TLRs (**Table 2**) and respond to their agonists by secreting cytokines, chemokines and lipid mediators which would have a profound effect on other cells of the immune system. In addition, TLR ligation can act to enhance the response of mast cells to antigen, sensitizing the cells to stimulation through FcεRI (Qiao et al., 2006; Fehrenbach et al., 2007; Hochdorfer et al., 2011). To date, the majority of the work investigating the function of TLRs has been performed *in vitro* with only a few studies *in vivo* (Supajatura et al., 2002; Mrabet-Dahbi et al., 2009). Further studies are therefore required to fully elucidate the role of TLR signaling in mast cells.

The signal transduction pathways triggered by TLR stimulation of mast cells are beginning to be elucidated and have some unique features. Strikingly, the MyD88-independent pathway which leads to IFN production is not induced by TLR4 activation, which may be due to a lack of cell surface CD14 (Keck et al., 2011). Indeed, the ability of mast cells to secrete IFN in response to other TLR stimulation is somewhat controversial (Kulka et al., 2004; Keck et al., 2011). In addition, some of the inhibitory pathways which have been identified in other immune cells are not observed to occur in mast cells (Hamerman et al., 2005; Zorn et al., 2009).

As described, several discrepancies are noted in the literature reporting the signaling pathways utilized by TLRs in mast cells and the response to TLR agonists. Notably, the ability of TLR2 ligation to induce degranulation is contentious, as is the ability of mast cells to respond to TLR3 ligation and the relative importance of the different MAPK proteins in TLR signal transduction. These differences may be explained by different mast cell culture conditions resulting in heterogeneous cell populations possibly with different expression of TLRs and signaling proteins. Further work is needed to consolidate the data. Given the importance of mast cells in the immune defense to bacteria and viruses (Marshall, 2004; Abraham and St John, 2010), it is important that the signal transduction pathways utilized by TLRs and the consequences of TLR signaling in these cells are understood.

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Novel identified receptors on mast cells

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Mast cells (MC) are major participants in the allergic reaction. In addition they possess immunomodulatory roles in the innate and adaptive immune reactions. Their functions are modulated through a number of activating and inhibitory receptors expressed on their surface. This review deals with some of the most recently described receptors, their expression patterns, ligand(s), signal transduction mechanisms, possible cross-talk with other receptors and, last but not least, regulatory functions that the MC can perform based on their receptor expression in health or in disease. Where the receptor role on MC is still not clear, evidences from other hematopoietic cells expressing them is provided as a possible insight for their function on MC. Suggested strategies to modulate these receptors' activity for the purpose of therapeutic intervention are also discussed.

Keywords: mast cells, activating receptors, inhibitory receptors, ligands, signal transduction, function, co-stimulation/cross-talk

BACKGROUND

Mast cells (MC) have the uniqueness of being tissue resident cells packaged with cytoplasmic granules full of preformed mediators of diverse nature, rich in surface receptors that upon ligand binding can induce not only the fast release of the stored mediators, but also the *de novo* synthesis of arachidonic acid metabolites and a number of chemokines and cytokines. Therefore they play a prominent role in maintaining homeostasis, acting as armed sentinel cells in the tissues, where they reside spanning from mucosal to connective tissues and more. MC have been historically associated with allergy, in which the key receptor is FcεRI. However, their strategic location and potential have clearly demonstrated that MC are more than the primum movens of allergic inflammation; they are very important players also in innate and adaptive immune responses, inflammation, and tissue changes. MC immunomodulatory roles may result in either negative or positive outcome for the host, enhancing, or suppressing certain features of immune/inflammatory responses. A large variety of receptors especially activating and some inhibiting MC functions, have been described. FcεRI, for IgE activation, and c-kit, the receptor of the stem cell factor (SCF), serve as fingerprints in MC characterization and are the topic of other reviews of this series.

Our purpose is to review herewith the expression pattern, function, ligand(s), and signal transduction pathways of some of the most recently described activating and inhibiting receptors identified to be expressed by the MC from different origins. We have mainly reviewed the novel activating receptors and discussed just a few examples of the inhibitory ones. The activating receptors are comprised of chemokines, interleukins (IL), amines, Toll-Like Receptors (TLRs), and others (**Table 1**). For some of the receptors, if known, the physiological and pathological consequences of their activation and the strategies to modulate their activity for the purpose of therapeutic intervention are also discussed. Evidences from other hematopoietic cells expressing these receptors is provided as a possible insight for their function on MC. Potential cross-talk between activating on one side and activating and inhibitory receptors (IRs) on the other side is also briefly considered.

CHEMOKINES RECEPTORS

Chemokines are small cytokine-like proteins that regulate leukocyte trafficking under homeostatic and inflammatory conditions (Luther et al., 2002; Homey and Bunemann, 2004; for chemokines receptors (CRs), CRs in MC, see review Juremalm and Nilsson, 2005). CRs, expressed on hematopoietic and lymphatic cells, are regulated by a variety of inflammatory stimuli (Olson and Ley, 2002). For example CXCR1 and CXCR2 are expressed on most leukocytes, but appear to be functionally significant only for neutrophils, monocytes/macrophages, and MC. CXCR3, CXCR5, and CXCR6 were found to be expressed on cells of lymphoid lineage (Olson and Ley, 2002). CXCR3 was later found to be expressed also by MC (Juremalm and Nilsson, 2005). A defined set of chemokines (CCL1–5, CCL11, CCL13, CCL17–18, CCL20, CCL22, CCL26–27, CX3CL1) was identified to initiate and perpetuate atopic skin inflammation (Homey et al., 2006, 2007).

Table 1 | Structural functional data, expression, ligand, and function of some MC receptors discussed in the review.

Receptor	Ligand	Structural functional data	MC expression	Function
Chemokines receptors	For CXCR3-CXCL9, CXCL10, and CXCL11 (Meiser et al., 2008)	GPCR	E.g., CXCR3 protein: human lung MC (Juremalm and Nilsson, 2005)	Migration (Juremalm et al., 2002), signaling events (Lacotte et al., 2009) partial degranulation (Willox et al., 2010)
IL-15R	IL-15	α Chain, β chain, γ chain	Protein: mBMMC Expression assumed by function: hCBMC (Jackson et al., 2005)	Migration (Jackson et al., 2005), enhancement of Th1 response, inhibition of allergic inflammation in a murine model of asthma (Ishimitsu et al., 2001)
IL-18R	IL-18	Dimeric complex of IL-18Rα, IL-18Rβ	mRNA and protein: mouse mucosal MC and immature MC (Wiener et al., 2008)	Production of IFN γ, GM-CSF, TNF-α and IL-1, IL-13, and/or IL-4 (Nakanishi et al., 2001; Akdis et al., 2011)
IL-33R (ST2)	IL-33	Complex: ST2 and IL-1RAcP	Protein: mBMMC (Liew et al., 2010)	Degranulation and proinflammatory cytokine production and release (Hsu et al., 2010; Liew et al., 2010)
TSLPR	TSLP	Complex: TSLPR with IL-7Rα	mRNA: mBMMC (Knisz et al., 2009). Protein: human peripheral blood and hCBMC (Allakhverdi et al., 2007)	Increasing of pro-inflammatory cytokines (Allakhverdi et al., 2007) production and Th2 response (Ziegler and Artis, 2010)
TLR	Bacterial and viral proteins	Extracellular leucine rich region, cytoplasmic domain that consists of three homologous regions (box 1, 2, 3)	TLR2 and TLR4 mRNA:immature and mature MC from murine and human origins (Supajatura et al., 2002). TLR2 protein: hCBMC and nasal polyps MC (McCurdy et al., 2003). TLR2 and TLR4 protein:murine intestine MC and murine and rat peritoneal MC (Pietrzak et al., 2011)	*de novo* synthesis and release of cytokines (McCurdy et al., 2001; Supajatura et al., 2002)
CD48	2B4, FimH, *S. aureus*, and *M. tuberculosis* toxins	Glycosyl-phosphatidyl-inositol (GPI)-anchored protein	Protein: BMMC and human peripheral blood MC	Allergic effector unit formation, stimulation of mediator release (Elishmereni et al., 2011)
S1P2R	S1P	GPCR	Protein: mBMMC and RBL-2H3 (Jolly et al., 2004), hMC (Oskeritzian et al., 2010), and hematopoietic progenitors (Price et al., 2009)	Degranulation (Wang et al., 2012) and chemokine and cytokine release (Oskeritzian et al., 2010) trafficking and migration (Spiegel and Milstien, 2011)
HR (H_1, H_2, H_3, H_4)	Histamine	GPCR	H1R mRNA and protein: (Lippert et al., 2004) low in human skin MC (Lippert et al., 2004; Gibbs and Levi-Schaffer, 2012), higher in HMC-1 cells	H1, H2-intracellular calcium mobilization (Tilly et al., 1990)
			H2R mRNA and protein: human skin MC, and HMC-1 cells (Lippert et al., 2004)	H3-autoregulation of histamine release (Ohkubo et al., 1994)
			H_3R protein: in brain mast cells (Rozniecki et al., 1999)	H4-chemotaxis and intracellular calcium mobilization in mMC (Nordlind et al., 2008), recruitment of effector cells in human (Hofstra et al., 2003)
			H4R mRNA: mouse MC, hCBMC, and HMC-1 (Hofstra et al., 2003; Gibbs and Levi-Schaffer, 2012), human skin MC (Lippert et al., 2004)	

(Continued)

Table 1 | Continued

Receptor	Ligand	Structural functional data	MC expression	Function
5HTR	Serotonin	GPCR	mRNA: mBMMC and human CD34+-derived MC (Kushnir-Sukhov et al., 2006)	Cell migration (Nordlind et al., 2008)
Purinergic P1	Adenosine	P1: GPCR	Adenosine receptors-A1protein: canine BR mastocytoma A2a protein: HMC-1, RBL-2H3, mBMMC A2b protein: canine BR mastocytoma, HMC-1, RBL-2H3, mBMMC A3 protein: mastocytoma, RBL-2H3, mBMMC (Forsythe and Ennis, 1999; Hua et al., 2011)	hCBMC implicating the A_1R in potentiation, and A_2BR receptor in the inhibition of anti-IgE-induced degranulation (Yip et al., 2009; Hua et al., 2011)
P2X	ATP	P2X: ion pore	E.g., $P2X_1$, $P2X_4$, $P2X_7$ protein: hCBMC(Bulanova and Bulfone-Paus, 2010). $P2X_{1–4}$, $P2X_{6–7}$ mRNA: mBMMC (derived from C57BL/6 mice)	Degranulation, apoptosis (Bulanova et al., 2009)
P2Y	ATP, ADP, UTP, UDP, UDP-glucose	P2Y: GPCR	P2Y receptors (with the exception of $P2Y_2$) protein: mBMMC $P2Y_1$, $P2Y_2$ $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{11}$: hCBMC (Feng et al., 2004)	Degranulation, $P2Y_{13}$ in RBL-2H3 Ca^{2+} release and release of β-hexosaminidase $P2Y_2$: mediation of chemotaxis in mBMMC and CD34+ progenitors. (Bulanova and Bulfone-Paus, 2010)
CD203c	NAD and nucleotide sugars	Type II-phosphatidyl-inositol transmembrane proteins with catalytic domain and a C-terminal endonuclease-like domain	mRNA and protein: overexpressed on neoplastic MC in patients with systemic mastocytosis (Hauswirth et al., 2008)	Cleavage of phosphodiester and phosphosulfate bonds (Buhring et al., 2004)
CRHR	CRH	GPCR	mRNA and protein: hCBMC and LAD-2 (Asadi et al., 2012)	Production and release of IL-8, TNF, VEGF. Induction of NK-1 gene expression (Asadi et al., 2012)
Endocannabinoids receptors	Endocannabinoids	GPCR	Protein: on human skin MC (Sugawara et al., 2012)	Inhibition of MC maturation and activation, regulation of SCF expression (Sugawara et al., 2012)

mRNA, messenger ribonuclear acid; MC, mast cell; IL, interleukin; GPCR, G protein coupled receptor; H, human; M, mouse; BMMC, bone marrow mast cells; CBMC, cord blood mast cells; IFN γ, interferon-gamma; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF α, tumor necrosis factor α; TSLPR, thymic stromal lymphopoietin receptor; TLR, Toll-like receptor; 5HTR, 5-hydroxytryptamine receptors; ATP, ADP, adenosine 5′-triphosphate, adenosine diphosphate; UTP, UDP, uridine triphosphate, uridine diphosphate; NAD, nicotinamide adenine dinucleotide; CRHR, corticotropin-releasing hormone receptor; CRH, corticotropin-releasing hormone; SCF, stem cell factor; S1P, sphingosine-1-phosphate; HR, histamine receptor.

Human MC (hMC) from different origins express at least nine CRs, i.e., CXCR1–4, CX3CR1, CCR1 (further detailed in MC Co-Stimulation), CCR3–5 that may be activated by the respective chemokines, i.e., CXCL1, CXCL5, CXCL8, CXCL14, CX3CL1, CCL5, and CCL11 (Juremalm and Nilsson, 2005). Interestingly, activation of CRs on MC can not only induce their chemotaxis, but also other stimulatory responses. For example, the activation of CXCR3 and CCR2, besides migration also induces human cord blood MC (hCBMC) signaling events or even partial degranulation in the absence of antigen (Willox et al., 2010). The expression of CXCR3 and RANTES (also known as CCL5) on MC is important especially in the migration of MC precursors in the tissues where they undergo final maturation (Juremalm et al., 2002). CCL5/RANTES can induce MC migration through interactions with CCR1 and CCR4 (Juremalm et al., 2002).

CXCR3 is a seven transmembrane (TM) domain G protein coupled receptor (GPCR) that binds the pro-inflammatory, non-ELR motif of CXC chemokines: CXCL9, CXCL10, and CXCL11 (Meiser et al., 2008) and was found to be sensitive to pertussis toxin (Ptx; Willox et al., 2010). Recently, two different variants of CXCR3 have been identified in human: CXCR3B and CXCR3-ALT, both originating from the same gene and produced by alternative splicing of the CXCR3 mRNA (Willox et al., 2010). CXCR3 activation leads to Gαi protein internalization with subsequent calcium influx

and triggering of mitogen-activated protein kinases (MAPK) and AKT (Protein kinase B, PKB) cascade resulting in cytoskeleton rearrangement and cell movement (Lacotte et al., 2009). Upon activation of this CR, cross-talk between the CXCR3 and FCεRI is possible due to the fact that both these receptors involve phosphoinositide 3-kinases (PI3Ks) signaling. CXCR3 is highly expressed in human lung MC that reside in proximity to the smooth muscle in asthmatics, but is not expressed in human bone marrow MC (hBMMC; Brightling et al., 2005). In human lungs, CXCR3 on MC mediates their migration toward chemokines-secreting airway smooth muscle regions. Additionally, CXCL10 has been shown to be expressed in that region in bronchial asthma biopsy specimens and *ex vivo* in smooth muscle cells from asthmatics compared to the ones derived from healthy volunteers (Okayama et al., 2008). Because of the importance of chemokines in recruiting inflammatory cells and MC precursors into the tissues, neutralization of chemokines, or antagonists of their receptors is an active investigational field in the pharmaceutical industry.

INTERLEUKIN RECEPTORS AND TSLPR

Interleukins are secreted glycoproteins that bind to their specific receptors and play a role in the communication among leukocytes. In the past decade, a number of new cytokines have been described such as IL-15, IL-18, IL-7, IL-33. Importantly, MC display the receptors for some of the newly identified IL.

THE IL-15 RECEPTOR

The classic IL-15 receptor (*IL-15R*) consists of a unique α chain, a β chain, and a common γ chain which is shared with the IL-2 receptor (Lorenzen et al., 2006). IL-15R signaling requires DAP10-associated proteins since the IL-15 signaling pathway phosphorylates DAP10 through JAK3. Once activated, IL-15R triggers the activation of JAK3-STAT5 signaling pathway that relays information to the nucleus (Colucci, 2007). IL-15R is expressed in B, T cells, and natural killers cells (NK) and its activation results in cell proliferation and differentiation. IL-15R was recently reported to be expressed on mouse BMMC (mBMMC). It has not yet been reported whether hCBMC express the same receptors. However, IL-15 induced migration of both mBMMC and hCBMC in a dose-responsive and biphasic manner, supports the possibility that this effect is mediated by two distinct IL-15R with different affinities to IL-15 (Jackson et al., 2005). Interestingly, in stimulated dendritic cells (DC), IL-15, and IL-15Rα are preassembled in complexes within the endoplasmic reticulum/Golgi before being released from the cells. In NK, membrane bound IL-15-IL-15Rα complexes, and not the soluble ones, trigger activation (Mortier et al., 2008).

The ligand of IL-15R, IL-15, is produced in human by both non-immune cells (keratinocytes and skeletal muscle cells) and by immune cells (monocytes, and activated CD4+ T cells) in response to signals that induce innate immunity (Akdis et al., 2011). IL-15 is also produced by murine MC, especially after stimulation with lipopolysaccharide (LPS; Orinska et al., 2007). IL-15 in MC induces an intracrine signal that downregulates chymase-dependent, MC-mediated innate immunity (Orinska et al., 2007). In *Il-15*$^{-/-}$ mice, the absence of IL-15 in MC increases chymase activities, leading to greater MC bactericidal responses, increased processing, and activation of neutrophil-recruiting chemokines, and improved sepsis survival (Orinska et al., 2007).

IL-15 is associated with autoimmune and inflammatory diseases and was recently shown to be upregulated in T cell-mediated inflammatory disorders, such as rheumatoid arthritis (RA) and inflammatory bowel diseases (Waldmann, 2004). In rheumatoid synovial tissue explants, αIgE induced MC activation induces changes in the amounts of released tryptase, TNFα, and IL-1β by a proportion of mononuclear inflammatory cells, but not in the amount of IL-15 (Woolley and Tetlow, 2000). IL-15 is also associated with the development of X-linked severe combined immunodeficiency (Akdis et al., 2011). Overexpression of IL-15 *in vivo* enhances Th1 responses, which inhibit allergic inflammation in a murine model of asthma (Ishimitsu et al., 2001). In a human psoriasis xenograft model, the anti IL-15 antibody 146B7 was found to be involved in the reduction of the severity of the disease (Villadsen et al., 2003). Additionally, HuMax-IL-15, a human IgG1 anti-IL-15 monoclonal antibody, that *in vitro* was able to neutralize exogenous and endogenous IL-15, has been used for clinical trials in patients with RA to perform a phase I–II dose-escalation trial (Baslund et al., 2005; Waldmann, 2006).

THE IL-18 RECEPTOR

IL-18 receptor (IL-18R) expressed on MC belongs to the IL-1 receptor (IL-1R) family (Thomassen et al., 1998). IL-18Rα is the binding chain and together with IL-18Rβ form a high affinity heterotrimeric complex with the ligand IL-18. This complex recruits the intracellular adapter molecules myeloid differentiation primary response protein 88 (MyD88), IL-1 receptor-associated kinases (IRAK) and tumor necrosis factor receptor associated factor 6 (TRAF6) which results in the activation of p38 MAPK, JUN N-terminal kinase (JNK), and/or nuclear factor kB (NF-kB; Arend et al., 2008). IL-18R is expressed on T cells, NK cells, macrophages, epithelial cells, chondrocytes (Kunikata et al., 1998), and MC (Helmby and Grencis, 2002).

The ligand of IL-18R, IL-18, is an IFN-γ-inducing factor and a potent cytokine that is produced by monocytes/macrophages as a reaction to different microbial components and therefore plays a major role in the innate immune responses to pathogens (Arend et al., 2008). Also Kupffer cells, keratinocytes, osteoblasts, astrocytes, and DC express IL-18 (Akdis et al., 2011). The regulation of IL-18 biological activity is carried out by caspase-1-mediated cleavage of pro-IL-18 to the mature protein. Human chymase can also cleave pro-IL-18 to the active protein (Omoto et al., 2006). IL-18 binding protein (BP) is a soluble protein that by binding mature IL-18 prevents its binding to receptor, and therefore serves as a natural inhibitor (Novick et al., 1999). IL-18BP displays four isoforms in humans and two in mice that are the result of alternative splicing of mRNA and which mainly differ in the C-terminal region. IL-18 indirectly increases the production of its own inhibitor in a feedback loop via INFγ secretion. IL-18R activation is able to induce in addition to IFNγ, also GM-CSF, TNFα, and IL-1 production. In addition it can induce IL-13 and/or IL-4 production by NK, MC, and basophils. It can therefore enhance innate immunity and both Th1 and Th2 driven immune responses (Nakanishi et al., 2001).

IL-18 has been associated with autoimmune diseases or inflammatory disorders, bronchial asthma (Harada et al., 2009), atopic

dermatitis (Konishi et al., 2002), RA (Gracie et al., 1999; Plater-Zyberk et al., 2001), psoriasis (Ohta et al., 2001), multiple sclerosis (Karni et al., 2002), and type I diabetes (Altinova et al., 2008; Akdis et al., 2011). Regarding allergy, interestingly IL-18 contributes to the spontaneous development of IgE/signal transducer and activator of transcription (STAT6)-independent atopic dermatitis-like inflammatory skin lesion (Konishi et al., 2002).

In conclusion, IL-18/IL-18R regulation, if carried out by selective antagonists, can possibly be considered as a good pharmacological target in either Th1 or Th2 driven conditions.

IL-33 RECEPTOR

IL-33 receptor (*IL-33R*) termed also *ST2* is a member of the IL-1R family, and TLR/IL-1R (TIR) superfamily (Liew et al., 2010). It binds IL-33 as a heterodimer consisting of ST2 and IL-1R accessory protein. The signaling cascade of ST2 activation involves recruitment of MyD88, TRAF6, and IRAK. This leads to NF-kB activation and the activation of MAPK p38, signal-regulated kinase ERK, and JNK. ST2 is expressed on basophils, MC, eosinophils, NK cells, Th2 cells, DC, and nuocytes (Kunikata et al., 1998; Akdis et al., 2011).

Mouse BMMC express high levels of ST2 and respond directly to IL-33 to produce a spectrum of inflammatory cytokines and chemokines (IL-1, IL-6, IL-13, TNF, CCL2, and CCL3; Liew et al., 2010). *In vivo*, IL-33 treatment exacerbated collagen-induced arthritis in $ST2^{-/-}$ KO mice engrafted with MC from WT but not from $ST2^{-/-}$ mice, a fact that was associated with elevation in expression levels of pro-inflammatory cytokines (Xu et al., 2008). The ST2 ligand, IL-33, is a pro-inflammatory cytokine that activates Th2 response-inducing cells. IL-33 activation induces mBMMC and the murine MC line MC/9 proliferation and Th2 cytokine production including IL-4, IL-5, IL-6, but not IL-33 itself (Hsu et al., 2010). On the other hand mBMMC produce IL-33 in response to IgE activation, which requires calcium influx or ionomycin activation (Hsu et al., 2010).

IL-33/ST2 pathway is critical for the development of IgE-driven tissue inflammation during passive cutaneous anaphylaxis that is a strictly MC-dependent model (Hsu et al., 2010). Levels of soluble ST2 increase in inflammatory conditions such as systemic lupus erythematosus (SLE), RA (Xu et al., 2008), idiopathic pulmonary fibrosis (Tajima et al., 2003), asthma (Oshikawa et al., 2001), progressive systemic sclerosis, Behcet's disease, Wegener's granulomatosis, severe trauma, and sepsis (Akdis et al., 2011). Importantly, in all of these conditions MC activation and role have been illustrated. IL-33 release is elevated in skin of patients with atopic dermatitis (Liew et al., 2010). Constant mechanical microtrauma and destruction of skin barrier induce IL-33 release and a pro-inflammatory response. In addition, IL-33 may play a role in psoriasis-like plaque inflammation (Hueber et al., 2011).

IL-33 has been classified as an alarmin/danger signal (Cevikbas and Steinhoff, 2012) since it is released by cells undergoing necrosis and it is inactivated by caspases during cell apoptosis (Liew et al., 2010; Hueber et al., 2011). However, it is evident that IL-33 is released not only from necrotic cells, but also from living cells and therefore acts as a classical cytokine (Liew et al., 2010). Its importance in allergy is the current topic of high interest partly due to its effects on the MC.

TSLP RECEPTOR

TSLP receptor (*TSLPR*) is the receptor of TSLP that is an IL-7-like cytokine initially identified in the culture supernatant of a thymic stromal cell line (He and Geha, 2010). TSLPR has a low affinity to TSLP, but together with IL-7Rα they generate high affinity binding sites and trigger signaling (Park et al., 2000). Cross species homology of TSLP and its receptor is relatively low (about 40%; He and Geha, 2010; Ziegler and Artis, 2010), but the fact that both in human and in mouse IL-7Rα is required suggests that human TSLP and TSLPR are orthologs to mouse TSLP and TSLPR. The signaling cascade of these receptors is not completely clear and no Janus kinases (JAK) are activated (Isaksen et al., 2002). It has however been shown that receptor engagement can activate the transcription factor STAT3 in human and STAT5 in mouse and human (Isaksen et al., 2002). In addition, it can also induce the expression of common genes (such as *Cish*; Isaksen et al., 2002).

TSLP receptor is expressed on hematopoietic cell lineages, including B cells, T cells, MC, eosinophils, and DC (Taylor et al., 2009). mBMMC were shown to express TSLPR mRNA (Knisz et al., 2009). TSLPR and IL-7Rα chain expression were determined at the mRNA level on human peripheral blood and hCBMC (Allakhverdi et al., 2007). In spite of these reports, human peripheral blood derived MC (hpbMC) and the HMC-1 cell line (hMC leukemia-1) were found by us to express TSLPR, but not IL-7R (Levi-Schaffer, F., Soumelis, V., Levy, I., unpublished data). At the protein level, TSLPR was also reported to be expressed *in vivo* on MC infiltrating the bronchial mucosa of asthmatic patients, as revealed by immunostaining of biopsy specimen (Allakhverdi et al., 2007; Comeau and Ziegler, 2010; Shikotra et al., 2012).

It has been found that MC infiltrating to the mucosal gland stroma and airway smooth muscle in asthma not only express high levels of TSLP mRNA, but can also respond to TSLP (Rochman and Leonard, 2008). TSLP can directly activate hMC to produce pro-inflammatory Th2 cytokines and chemokines in the presence of IL-1β and tumor necrosis factor α (TNF-α), in a way that mimics inflammation conditions (Allakhverdi et al., 2007). Various cell types can produce TSLP: endothelial, epithelial cells and epidermal keratinocytes, airway smooth muscle cells, fibroblasts, DC, trophoblasts, and cancer or cancer-associated cells (Takai, 2012). hMC express TSLP mRNA, which is upregulated upon cross-linking of the IgE receptor. Pre-incubation with IL-4 results in significant upregulation of IgE-mediated TSLP protein and mRNA expression (Okayama et al., 2009; Comeau and Ziegler, 2010).

TSLP plays a significant role in initiation of allergic inflammation and is very important to cells such as the MC, due to its high expression in the interfaces between the body and environment and its ability to lead to Th2 responses (Ziegler and Artis, 2010). Different studies have shown that asthma, allergic rhinitis, and atopic dermatitis are characterized by an increased expression of TSLP in the inflamed tissue (Ziegler and Artis, 2010; Le et al., 2011). Mice overexpressing TSLP on $TCR\beta^{-/-}$ background, develop dermal inflammation and skin infiltrates of MC and eosinophils (Yoo et al., 2005). MC deficient mice failed to upregulate TSLP in nasal epithelium after allergen challenge in a model of allergic rhinitis (Ziegler and Artis, 2010). In asthma it was demonstrated that MC play an important role in TSLP production (Shikotra et al., 2012). TSLP levels are increased in human asthma and correlate with the

increase in expression of Th2 cytokines and disease severity (Ying et al., 2005, 2008; Corrigan et al., 2009; Fang et al., 2010).

Blocking of TSLP signaling using TSLPR-immunoglobulin in murine asthma model, was shown to regulate pulmonary DC function and to reduce eosinophilic airway inflammation and Th2 differentiation significantly (Zhang et al., 2011). Additionally, TSLP/TSLPR interaction influences the function of cells in host protection against helminth parasites, and modulates gut homeostasis in conditions in which MC are involved (Comeau and Ziegler, 2010). Therefore it appears that MC displaying TSLPR and producing TSLP have a potent weapon in addition to FcεRI in orchestrating the allergic reactions. As a consequence the TSLP axis is a very promising candidate for anti-allergic intervention.

Notably, TSLP and IgE stimulated MC can induce OX40 ligand (OX40L) expression on DC. (Ito et al., 2005; Edwards, 2008). OX40 receptor and its ligand OX40L belong to the superfamilies TNF receptor and TNF respectively (Godfrey et al., 1994) and were shown to have a crucial role in allergic inflammation. OX40/OX40L interaction can trigger the differentiation of some inflammatory Th2 cells in the lymph nodes. (Liu, 2007; Wang and Liu, 2007) OX40L on MC and OX40 on T regulatory cells cross-talk can induce T cells activation by hMC *in vitro* (Kashiwakura et al., 2004) and inhibit FcεRI-dependent MC degranulation both *in vitro* and *in vivo* (Gri et al., 2008). Recently, Ilves and Harvima (2012) showed that there are more OX40 positive cells in the dermis from AD lesions than in healthy looking dermis, which does not correlate with clinical severity of AD.

TOLL-LIKE RECEPTORS AND OTHER BACTERIAL AND VIRAL RECEPTORS

As key protagonists of innate immunity, MC play a pivotal role in anti-infection defense and "danger" responses. MC reactivity against bacteria have been more characterized than the ones against viruses, although both of them are classically mediated by TLRs (Akira and Takeda, 2004), a family of pattern recognition molecules.

Toll-like receptors and IL-1R have a conserved cytoplasmic domain that consists of three homologous regions (boxes 1, 2, 3) and an extracellular domain that differs: TLRs have tandem repeats of leucine rich regions, while IL-1R has three immunoglobulin like domains (Akira and Takeda, 2004). Stimulation of TLR triggers the association of MyD88, IRAK4, and IRAK1 signaling leading to the NF-kB activation and gene expression (Akira and Takeda, 2004). MC signaling via TLRs involves also ITAM-containing molecule DAP12 phosphorylation that activates syk, which is a critical molecule of MC activation (Smrz et al., 2010). This would suggest a cross-talk between TLRs and receptors that employ DAP12 as a transducer molecule.

TLR2 and TLR4 expression has been detected at the mRNA level in immature and mature MC from murine and human origins (Supajatura et al., 2002). At the protein level, TLR2 was found to be expressed in hCBMC and in nasal polyps MC (McCurdy et al., 2003). TLR2 and TLR4 are found in murine intestine MC and murine and rat peritoneal MC (Pietrzak et al., 2011). TLR signaling events induce cytokine production (Supajatura et al., 2002). TLR2 can be activated by prolonged stimulation with bacterial wall components, such as LPS and peptidoglycan (PGN), resulting in *de novo* synthesis and release of various cytokines. It was shown that PGN was able to induce degranulation of mBMMC via TLR2 accompanied by Ca^{2+} influx, whereas the activation of MC by LPS via TLR4 did not lead to degranulation (Supajatura et al., 2002). Cytokine exposure of MC can also upregulate the expression of TLRs. For example, IL-6 treatment of mMC induces an increase in TLR4 expression, whereas exposure to TNF does not influence the TLR2 and TLR4 protein levels (McCurdy et al., 2001). On the contrary, exposure of MC to CCL5 resulted in decreased expression of both TLR2, following 24 h incubation, and TLR4 level, following 12 and 24 h incubation (McCurdy et al., 2001).

CD48

CD48, a CD2-like molecule, is a 40KD glycosyl-phosphatidylinositol (GPI)-anchored protein, expressed on the surface of hematopoietic cells (recently reviewed in Elishmereni and Levi-Schaffer, 2011). The CD48 structure combines a distal V-like domain with a C2-like domain containing conserved cysteine residues that form disulfide bonds. It lacks a TM domain and is attached to the cell surface by a glycolipid, GPI, restricted to the outer leaflet of the membrane bilayer (Shin and Abraham, 2001). CD48 is found also in soluble form, due to its cleavage upon activation. Stimulated CD48 associates to the kinase LCK and leads to tyrosine phosphorylation (Elishmereni and Levi-Schaffer, 2011).

CD48 is illustrated here since in MC, CD48 was first described to be involved in innate immunity as it binds FimH of *E. coli*. This interaction triggers a strong TNF-α release and the uptake of this Gram-bacteria (Malaviya et al., 1999; Malaviya and Abraham, 2001; Proft and Baker, 2009). CD48, has also been implicated in MC interactions with *M. tuberculosis*, which triggers the release of several pre-stored mediators, such as histamine and β-hexosaminidase, and the *de novo* synthesis of cytokines, such as TNF-α and IL-6 (Munoz et al., 2003). Later on we found that *S. aureus*, a Gram+ bacteria, and its toxins bind to CD48 and TLR2 on hCBMC causing subsequent release of pro-inflammatory cytokines (Rocha-de-Souza et al., 2008). CD48 is overexpressed in murine asthma and was defined as a signature gene in this condition (Zimmermann et al., 2003). The CD48 ligand in human is 2B4, expressed by several hematopoietic cells. CD48/2B4 interactions taking place between MC and eosinophils are important in allergic inflammation giving rise to the physical formation of the allergic effector unit (AEU) between these two cells (Elishmereni et al., 2011). CD48-2B4 binding induces degranulation of MC and increases eosinophil survival and activation (Elishmereni et al., 2011). In a murine model of allergic asthma, treatment with neutralizing CD48 Ab dramatically inhibited the lung inflammation (Munitz et al., 2007). Therefore it seems that CD48 is a good candidate to be blocked on MC in order to avoid both AEU formation and bacterial invasion, with this last property being particularly helpful in allergic conditions, such as atopic dermatitis, that are commonly associated with *S. aureus* infection.

S1P RECEPTORS

S1PR$_{1–5}$ are GPCRs of sphingosine-1-phosphate (S1P). Secretion of S1P by MC can modulate their function by binding to $S1PR_1$ and $S1PR_2$ found on MC, in an autocrine fashion (Spiegel and Milstien, 2011). It was shown on DC that there is a cross-talk

between $S1PR_2$ and $S1PR_1$, when upregulation of $S1PR_2$ activates the small GTPase RHO leading to translocation of Four and a Half LIM domains protein 2 (FHL2) to the nucleus and downregulates $S1PR_1$ expression. On the other hand, downregulation of $S1PR_2$ increases $S1PR_1$ expression and RAC activation (Spiegel and Milstien, 2011).

$S1P_1$ is involved in the migration of MC toward low concentrations of antigen, while $S1P_2$ participates in FcεRI-induced degranulation (Jolly et al., 2005; Olivera, 2008). S1P released from MC after cross-linking of FcεRI, and their $S1P_2$ receptors are critical for degranulation and chemokine, cytokine, and lipid mediator release from activated human and rodent MC (Oskeritzian et al., 2010). Additionally, blocking of the S1P–$S1PR_2$ axis was shown to drastically reduce circulating levels of histamine in a mouse anaphylaxis model indicating that MC were inhibited (Oskeritzian et al., 2010). The $S1PR_2$ activation plays a role in anti-viral immunity: the viral membrane lipid sphingomyelin that is converted in the cell membrane to S1P can then activate the $S1PR_2$ in an autocrine manner to stimulate MC degranulation (Wang et al., 2012). S1P–$S1PR_1$ axis has been shown to be expressed on and to control the trafficking and migration of numerous types of immune cells, including T and B lymphocytes, natural killer T cells, DC, macrophages, neutrophils, hematopoietic progenitors, MC, and osteoclasts (Spiegel and Milstien, 2011).

Therefore, S1PR can offer an interesting pharmacological target for a number of immune diseases. For example the $S1PR_2$ antagonist JTE013 significantly inhibited H_2O_2-induced permeability in the rat lung perfused model (Sanchez et al., 2007). More studies are under way to exploit the blockage of the S1P and its receptors in many diseases and notably in allergy.

AMINES' RECEPTORS

Among the mediators extensively identified with the function of MC in allergy, histamine in humans and rodents, and serotonin especially in rodents are key factors influencing the activity of a number of target cells/organs. MC not only produce these two potent amines but also display some of their receptors (Gibbs and Levi-Schaffer, 2012; Ritter et al., 2012).

HISTAMINE RECEPTORS

Histamine receptors (*HRs*) are GPCRs with seven TM-spanning helices. Until now, four subtypes of human HRs have been identified: H1R, H2R, H3R, and H4R. H1R and H2R have been the focus of many studies (recently reviewed in Gibbs and Levi-Schaffer, 2012). Fewer studies have been performed on the H3R and even less on the newest identified one, H4R. Regarding HRs expression by MC, not much work had been carried out until the recent discovery of H4R.

H1R expression has been described on human skin MC to be low (Lippert et al., 2004; Gibbs and Levi-Schaffer, 2012), while on the HMC-1 cells it is higher (Lippert et al., 2004). H1R antagonists can inhibit MC activation and therefore can be used as anti-allergic/MC stabilizing drugs (Levi-Schaffer and Eliashar, 2009), even though some of them might increase cellular cAMP in competitive antagonism with H2R. In cholangiocytes, H1R acts by Gαq activating IP(3)/Ca^{2+}, whereas H2R activates Gα(s) stimulating cAMP (Francis et al., 2012). Additionally, it was reported that H2R can activate both adenylate cyclase and phospholipase C signaling pathways via separate GTP-dependent mechanisms (Wang et al., 1996). Interestingly, via the H2R, histamine displays a net inhibitory action on MC (Gibbs and Levi-Schaffer, 2012). H2R is expressed on human skin MC, on HMC-1 cells and basophils, and perhaps on human lung MC. The H2 agonist impromidine was shown to inhibit MC activated with compound 48/80 and this inhibition was reversed by the histamine antagonists (Masini et al., 1982).

H3R and H4R have about 40% sequence homology and are functionally related (Morse et al., 2001; Zhu et al., 2001). Both of them are activated by α-methylhistamine (H3/4R agonist) and inhibited by thioperamide (antagonist). Mouse MC were reported to express H4R and to lack H3R expression (except brain MC that do express H3R; Pillot et al., 2002; Gibbs and Levi-Schaffer, 2012). In mouse MC, the H4R has been shown to couple to Ca^{2+} mobilization, but not to cAMP, in a Ptx-sensitive manner (Hofstra et al., 2003; Rosethorne and Charlton, 2011). H4R in mouse MC affects chemotaxis and intracellular calcium mobilization, and although it is not involved in their degranulation, the receptor activation and subsequent histamine release leads to recruitment of effector cells, especially eosinophils, rich in H4R to the site of chronic allergic inflammation (Hofstra et al., 2003). H4R activation of murine MC increases leukotriene B4 (LTB4) release and supports neutrophil recruitment induced by zymosan (Takeshita et al., 2003). H4R blockade was shown to decrease MC and eosinophil migration to the airway epithelial tissue after guinea pigs allergen exposure (Yu et al., 2008). Blocking the H4R also decreases the MC migration in presence of CXCL12 *in vitro* (Godot et al., 2007). Therefore, H4R plays a role in allergy by mediating the recruitment of cells to the site of allergic inflammation and also by controlling this last effect together with H1R. It has recently been demonstrated that in a mouse model of allergic pruritus, the H4R antagonist JNJ7777120 is more effective in reducing the response to histamine release than H1R antagonist (Dunford et al., 2007). It is foreseen that combinatorial therapy with both H4R antagonists – when available for human use – to target mostly MC and eosinophils and H1R antagonists could be a potent new therapy for some allergic responses rather than just H1R antagonists.

SEROTONIN RECEPTORS (5HTRs)

Serotonin is a neurotransmitter that can modulate a number of functions also outside the neural system (Berger et al., 2009). Serotonin receptors, also known as 5-hydroxytryptamine receptors (*5HTRs*), are classified in humans into seven main families with an additional subtype for two of the families (IUPHAR Receptor Database, 2008). Although 5HTRs have been known for a long time they have only recently been characterized on MC. With the exception of the 5HTR3, a ligand gated ion channel, all other serotonin receptors are GPCRs (Ritter et al., 2012) that activate an intracellular second messenger cascade to produce an excitatory or inhibitory response.

5HT1AR is negatively coupled to adenyl cyclase via Gi and it is expressed on hMC (Kushnir-Sukhov et al., 2006; Ritter et al., 2012) and mBMMC (Kushnir-Sukhov et al., 2006). It is expressed on MC in normal skin and its activation induces inflammation, due to MC migration and adherence in the site of inflammation, but it does not involve degranulation (Nordlind et al., 2008). Even though a still controversial issue, hMC were found to be able to secrete

serotonin both in IgE-dependent and independent manner (Askenase et al., 1991; Matsuda et al., 1995). This was further shown to activate effector T cells and macrophages (Young and Matthews, 1995), and also could show the possibility of an autocrine regulation of the MC. 5HT/5HT1AR are involved in allergic contact eczema, atopic eczema, psoriasis (Nordlind et al., 2008), and also expressed in human mastocytosis (Ritter et al., 2012).

PURINERGIC RECEPTORS AND ATP-HYDROLYZING ENZYMES

PURINERGIC RECEPTORS

Purinergic receptors, also known as purinoceptors, are a family of plasma membrane molecules involved in several cellular functions such as vascular reactivity, apoptosis, and cytokines secretion. The term, *purinergic receptor* was originally introduced to illustrate specific classes of membrane receptors that mediate relaxation of gut smooth muscle as a response to the release of Adenosine 5′-triphosphate (ATP) – P2R receptors or adenosine – P1R (King and Burnstock, 2002). There are three known distinct classes of purinergic receptors, referred to as P1, P2X, and P2Y receptors comprehending different subtypes (Bulanova and Bulfone-Paus, 2010). P2Y receptors, in addition to ATP, respond also to different nucleotides (ADP, UDP, UTP, UDP, and UDP-glucose). P1 and P2Y are GPCRs whereas P2X is a ligand gated ion channel.

Mast cells from most species express P1Rs: A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (Hua et al., 2011). Additionally they express a large variety of P2R. hCBMC, for example, express $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$ (Bulanova and Bulfone-Paus, 2010), $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{11}$ (Feng et al., 2004), while mBMMC (derived from C57BL/6 mice) express the mRNA of $P2X_{1–4}$, $P2X_{6–7}$, and all P2Y receptors with the exception of $P2Y_2$. P815 mastocytoma cells (derived from DBA/2 mice) express mRNA for all P2X and P2Y receptors (Bulanova and Bulfone-Paus, 2010). Activation of P2XR leads to the formation of a non-selective cationic channel (Burnstock, 2007), that triggers the activation of a number of intracellular signaling molecules, including MAPK, which is connected to cytokine secretion and release. ATP induces purinergic receptor $P2X_7$ mediated membrane permeabilization, apoptosis, and cytokine expression in murine MC (Bulanova et al., 2009).

Stimulation of P2YR generally leads to phospholipase C (PLC) activation that cleaves PI(4,5)P2 to form IP3 and diacylglycerol, an activator of protein kinase C (PKC). Only the $P2Y_{11}$ receptor is directly coupled to activation of adenylate cyclase (AC) and PLC, while $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors negatively affect cAMP synthesis (Van Kolen and Slegers, 2006). Extracellular nucleotides activating P2 receptors are involved in the regulation of MC degranulation. $P2Y_{13}$ receptor in a rat MC line (RBL-2H3) activation by ADP leads to intracellular calcium mobilization and release of β-hexosaminidase. Nucleotides via $P2Y_2$ mediate chemotaxis in mBMMC and CD34+ progenitors (Bulanova and Bulfone-Paus, 2010).

ATP acts as an autocrine and paracrine factor that enables the intercellular communication. MC IgE-dependent degranulation induces the release of ATP that influences the neural cells via P2X and P2Y receptors (Bulanova and Bulfone-Paus, 2010).

A study recently reported by Yip et al. showed a biphasic effect of adenosine in hCBMCs, implicating the A_1R in potentiation, and A_2BR receptor in the inhibition of anti-IgE-induced degranulation (Yip et al., 2009; Hua et al., 2011). Interestingly, also leukotriene E4 acts as ligand for $P2Y_{12}$ in LAD-2 cells. In addition it can induce purinergic receptor mediated pulmonary inflammation in mice (Paruchuri et al., 2009). It has been shown that adenosine binding to P1R on airway smooth muscle, goblet cells, MC, and neurons contributes to the pathogenesis of asthma (Okayama et al., 2008; Vieira et al., 2011). Adenosine inhibits and potentiates IgE-dependent histamine release from human lung MCs by an A_2-purinoceptor mediated mechanism (Hughes et al., 1984).

Xanthine derived drugs such as theophylline that have been widely used for asthma, have been found to be both antagonists/agonists of adenosine receptors and phosphodiesterase inhibitors on MC. Several antagonists are currently available for research use, such as suramine (Gever et al., 2006). Although the purinergic receptors are well described in the nervous system, their discovery on MC is still relatively new and further research is required in order to understand their specific function and effects on these cells.

CD203c

CD203c (E-NPP3) belongs to a family of ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) that catalyzes the cleavage of phosphodiester and phosphosulfate bonds of a variety of molecules, including deoxynucleotides NAD and nucleotide sugars (Buhring et al., 2004). These type II TM proteins are composed of a short N-terminal cytoplasmic domain, followed by the TM region, two somatomedin-like domains, the catalytic domain and a C-terminal endonuclease-like domain. Recently, CD203c has been defined as a novel activation-linked surface antigen on MC that is upregulated in response to IgE receptor cross-linking and is overexpressed on neoplastic MC in patients with systemic mastocytosis (Hauswirth et al., 2008). CD203c on basophils serves as a marker to diagnose various allergic diseases such as asthma (Ono et al., 2010). The expression and role of ATP-hydrolyzing enzymes for the biology of MC is still under investigation.

CORTICOTROPIN-RELEASING HORMONE AND ENDOCANNABINOIDS

Even though Corticotropin-releasing hormone (CRH) and endocannabinoids receptors have been typically described as central nervous system (CNS) receptors involved in regulation of the hypothalamic–pituitary–adrenocortical axis and in drugs response (Goeders and Guerin, 2000; Steiner and Wotjak, 2008), these receptors have also been found to be expressed on hematopoietic cells thus regulating peripheral conditions.

CORTICOTRICOTROPIN RELEASING HORMONE RECEPTORS

Corticotricotropin releasing hormone receptors (*CRHRs*) are GPCRs which are activated by urocortin (UCN), CRH, or substance P and consist of two receptors-CRH-R1 and CRH-R2, each encoded by a separate gene (Theoharides et al., 1998; Slominski et al., 2001; Asadi et al., 2012). CRHR signaling involves cAMP/PKA pathway or alternatively MAPK or intracellular calcium (Ca^{2+})/PKC pathways, depending on the site of activation (Grammatopoulos, 2011).

CRH-R1 is sensitive to β-arrestin-dependent signaling desensitization and internalization. Receptor endocytosis is also one of

the mechanisms employed by CRH-R1 to induce ERK1/2 and p38 MAPK phosphorylation and activation intermediates (Markovic et al., 2008). CRH-R1 variant is expressed in a tissue-specific manner; it is present in anterior pituitary as well as in peripheral sites such as reproductive tissues (myometrium, endometrium, and chorion trophoblast cells) and MC (Nezi et al., 2010).

Corticotropin-releasing hormone is secreted under stress and activates the hypothalamic–pituitary–adrenal axis. Interestingly a similar network is found in the skin (Asadi et al., 2012). Addition of CRH to the LAD-2 (leukemia MC line cells) primed with substance P induces synthesis and release of IL-8, TNF, and vascular endothelial growth factor (VEGF) 24 h later (Asadi et al., 2012). MC activation by CRH induces gene expression of neurokinin (NK-1). The ability of CRH to activate MC may explain its pro-inflammatory actions and the pathophysiology of certain skin conditions, which are precipitated or exacerbated by stress, such as atopic dermatitis, eczema, psoriasis, and urticaria (Theoharides et al., 1998). CRH has pro-inflammatory actions not only on its own, but also by augmenting the expression of the respective receptors on human skin MC (Asadi et al., 2012).

ENDOCANNABINOIDS RECEPTORS

The *cannabinoid receptors* are a class of cell membrane receptors belonging to the GPCRs superfamily. They contain seven TM spanning domains and are divided into two subtypes, termed cannabinoid 1 (CB1) and cannabinoid 2 (CB2). The endocannabinoid system has been recognized as a major neuromodulatory system, which functions to maintain brain homeostasis (Steiner and Wotjak, 2008). The CB1 receptor is expressed mainly in the brain CNS, but also in the lungs, liver, and kidneys. The CB2 receptor is expressed mainly in the immune system and in hematopoietic cells (Pacher and Mechoulam, 2011).

CB1 was found to be expressed on human skin MC (Sugawara et al., 2012) and be activated by endocannabinoids such as anandamide (AEA) and 2-arachidonoylglycerol. CB1 and CB2 receptors can induce inhibition of adenylate cyclase activity and phosphorylation with activation of p42/p44 MAPK, p38 MAPK, and JNK as signaling pathways to regulate nuclear transcription factors. The CB1 receptor regulates K^+ and Ca^{2+} ion channels, probably via Go/i (Howlett, 2005). A common role of CB1 and CB2 receptors appears to be the modulation of ongoing release of chemical messengers. The activation of CB2 receptors on MC has direct anti-inflammatory effects due to a decrease in mediators released from the cells (Pini et al., 2012).

The best CB2-selective agonists that have been developed so far include L-759633, L-759656, and JWH-133, all structural analogs of D9-THC. Other notable examples are the non-classical cannabinoid, HU-308, and the aminoalkylindole, AM1241 (Pertwee, 2006). The activation of CB1 receptor on bronchial nerve ending has a bronchodilatory effect (Pini et al., 2012). However, inhibiting the enzymatic inactivation of pharmacologically active endogenous molecules that do not serve as endocannabinoids or causing an accumulation of endocannabinoid molecules at non-CB1, non-CB2 targets such as the TRPV1 receptor or the putative abnormal-cannabidiol receptor, might lead to adverse effects.

Endocannabinoids limit excessive MC maturation and activation in human skin *in situ* and regulate SCF expression, by increasing its production in human hair follicle epithelium via CB1 stimulation (Sugawara et al., 2012). On the other hand in the ovalbumin induced lung sensitization model in CB1 and CB2 deficient mice a reduction in IgE production and attenuation of bronchoalveolar lavage fluid neutrophilia was described (Kaplan et al., 2010). In some studies, activation of CB1R seems to inhibit the allergic inflammation, while in others it is still controversial. Either way, we can conclude that CB1R activation is a potential target to be used in the treatment of allergy, although further research is still required.

MC RECEPTORS CROSS-TALK

Cross-talk between receptors might result in co-activation or in inhibition of cell response. Co-stimulation is often required for the development of an effective immune response, and has been recognized to participate in antigen-specific signal from lymphocytes antigen receptors. In the last few years, parallel to the identification of the new MC receptors, some cross-talk effects between receptors were described, resulting either in co-activation (synergism or additive effects) or in inhibition. Most of the works have dealt with the FcεRI mediated MC activation. For example IL-18R, IL-33R, TSLP, and TLRs were found to work via the same signaling pathway which activates MyD88, IRAK, TRAF, and MAPK and NF-kB (**Figure 1**). GPCRs on MC can also co-interact to produce different responses.

MC CO-STIMULATION

Due to co-stimulation and cross-talk significance in modulation of MC activity, we chose to review some recent examples of this phenomenon. CCRs are known stimulatory and co-stimulatory molecules expressed on MC. Fifadara et al. showed for the first time the formation of cytoneme-like cellular extensions by BMMC, a totally new feature of these cells, upon co-stimulation of FcεRI and CCR1 with antigen and CCR1 ligand, macrophage inflammatory protein-1α. This co-stimulation was more effective in some morphology changes and mediator release than FcεRI stimulation alone (Fifadara et al., 2010). Thus, CCR can contribute to the inflammatory and allergic responses by formation of cytonemes, as a way to communicate between MC and other cells. Recently, the same group has identified 32 genes that were differentially regulated by a co-stimulation of antigen and the CCR1 ligand CCL3, compared to stimulation with antigen or CCL3 alone, in RBL-CCR1 transfected cells. Four genes were mostly up regulated 3 h post co-stimulation (*Ccl7*, *Rgs1*, *Emp1*, *RT1-S3*), but only the CCL7 protein was expressed at a higher level 24 h following co-stimulation. Among chemokines and cytokines tested, only CCL2 protein showed higher expression levels and IL-6 was seen only after co-stimulation, although in a very low level (Aye et al., 2012). The authors suggested therefore that CCL2, CCL7, and IL-6 may be important for MC regulation in the late phase of the allergic response.

Another novel example of co-stimulation is the one between FcεRI and adenosine receptor. Nunomura et al. showed that co-stimulation of a low antigen dose together with adenosine can induce MC degranulation in a synergistic way, through cooperation of the two receptors mediated signaling. This was suggested to be relevant to the immediate asthma response upon bronchial

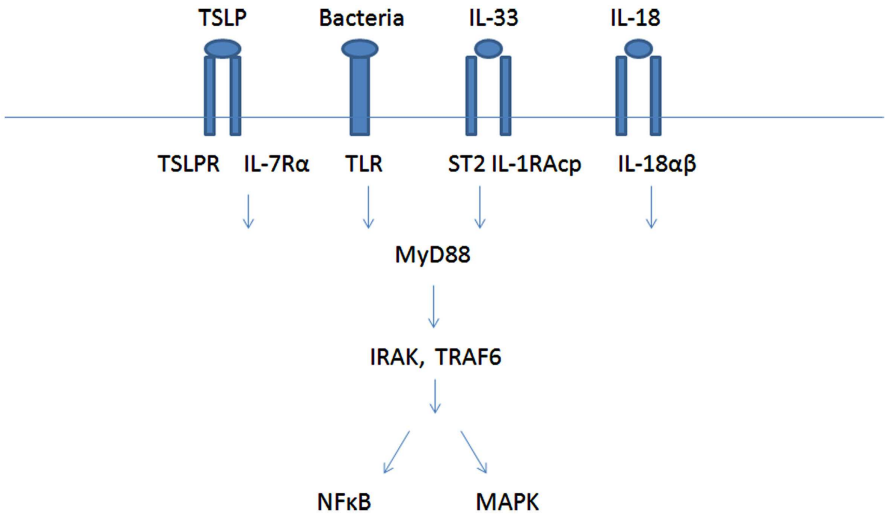

FIGURE 1 | Mast cells co-activation by IL-18, IL-33, TSLP, and TLR receptors.

challenge with low-dose allergen (Nunomura et al., 2010). It has also been shown that mBMMC mutant in the FcεRIβ-chain-ITAM could not be degranulated upon co-stimulation, through PI3K-signaling pathway (Nunomura et al., 2010). These findings can provide more detailed insight to which and how specific parts of the β chain and the PI3K-signaling are important for MC co-activation.

In mouse MC, thrombin, a serine protease, by binding to the protease-activated receptor (PAR)-1 TRAP(14) induces the secretion of IL-6 in a dose dependent fashion, but not of TNFα. Co-stimulation of low level thrombin with an allergen, can synergistically enhance IL-6 secretion through FcεRI signaling and the PI(3) and sphingosine-kinase pathways (Gordon et al., 2000).

Drube et al. reported a cross-activation of IL-33R and c-kit receptor in human and murine MC, after joining of IL-33R upon ligand binding, to a constitutively bound complex composed of c-kit and IL-1R accessory protein. In primary MC, the c-kit ligand SCF, is necessary for IL-33 induced cytokine production (Drube et al., 2010). Silver et al. showed that IL-33 in HMC-1 cells induced a synergistic activation effect together with adenosine, C5a, SCF, and NGF receptors. In primary hMC, IL-33R was also synergized with FcεRI activation (Silver et al., 2010).

CD48 was found to be a co-stimulatory receptor for inducing different effects in various hematopoietic cells by binding several ligands (Elishmereni and Levi-Schaffer, 2011). We have recently found that hCBMC and eosinophils can be directly activated by CD48 ligand/s (Elishmereni et al., 2011). In addition we detected some co-stimulatory effects when CBMC were activated by IgE-dependent mechanisms (Minai-Fleminger, Y., Levi-Schaffer, F., unpublished data).

CD84 is a self binding receptor from the signaling lymphocyte activating molecules (SLAM) family. However it was shown on transfected RBL-2H3 cells to inhibit FcεRI degranulation (Oliver-Vila et al., 2008). Additionally CD84 was found to be expressed on hMC (Alvarez-Errico et al., 2011), and shown to be tyrosine phosphorylated upon co-stimulation with FcεRI. This led to reduction in granule release and in the release of IL-8 and GM-CSF in LAD-2 cells and human CD34+ derived MC (Alvarez-Errico et al., 2011).

As reported in the past by our group, MC express DNAX accessory molecule 1 (DNAM-1, CD226) and eosinophils express its ligand Nectin-2 (CD112). It was shown that CD226 engagement with FcεRI induces an activating synergistic effect on MC and blocking of the ligand CD112 reduced the hyperactivity caused by IgE-dependent MC activation (Bachelet et al., 2006). To conclude, these few examples of co-stimulatory responses on MC can possibly help in understanding and foreseeing the complex allergic inflammatory response and be essential for the development of new anti-allergic therapy.

MC INHIBITORY RECEPTORS AND MC INHIBITION

Inhibitory receptors classically described on NK (Cantoni et al., 1999; for review, see Pegram et al., 2011) comprise two main families of the Ig receptor super-family and the c-type (calcium dependent) lectin superfamily. On MC, FcγRIIB, CD300, CD72, and sialic acid binding Ig-like lectins (Siglec)8, 7, etc., have been described (Karra and Levi-Schaffer, 2011). Since the FcγRIIB receptor has been extensively investigated (see Daeron et al., 2008; Bruhns et al., 2009) and we discussed some receptors bringing about MC inhibition, such as CB1, under their respective families, here we will consider only the most newly described typical IRs.

Inhibitory receptors expressed on MC can modulate their function by inhibiting typically the signaling from receptors associated with tyrosine kinases, i.e., FcεRI and c-kit (**Figure 2**). IRs contain immunoreceptor tyrosine based inhibition motifs (ITIMs) that down regulate the activation signals transmitted through immunoreceptor tyrosine based ligand ITAMs. The engagement of IRs suppresses cell activation by promoting dephosphorylation reactions. Upon activation of ITIM-containing receptors, tyrosine residues within the motifs become phosphorylated. This leads to the recruitment of protein phosphatases, Src homology 2 (SH) domain containing protein tyrosine phosphatase-1/2 (SHP-1 and SHP-2) and lipid phosphatases SH2 domain bearing

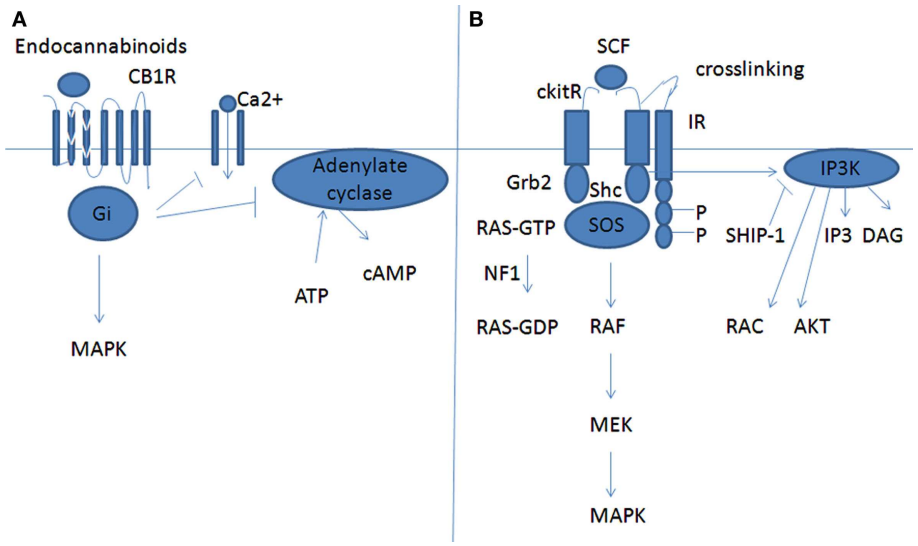

FIGURE 2 | Inhibition of MC maturation by CB1 or by a classical IR receptor. (A) CB1 inhibition effect. **(B)** Inhibition of c-kit mediated signaling by an inhibitory receptor (IR). Downstream molecules to CB1 or to c-kit cross-linked with the inhibitory receptor (cross-linking is mandatory to inhibitory effect).

inositol phosphatase (SHIP1). SHP-1/2 inhibits the action of tyrosine kinase, while SHIP1 terminates the PI3K-mediated pathway (Karra and Levi-Schaffer, 2011). Herewith we are going to illustrate the CD300a, Siglec 7 and 8, and CD72 IRs.

The CD300 family of myeloid immunoglobulin receptors includes activating (CD300b, CD300e) and inhibitory (*CD300a*, CD300f) members, as well as molecules of uncertain functions presenting a negative charge within their TM domain (CD300c, CD300d; Martinez-Barriocanal et al., 2010). None of the activating receptors identified contains a charged glutamic acid residue in the TM that is present in CD300c, suggesting that CD300c might deliver activating signals via signaling proteins other than those previously observed or that CD300c is not an activating receptor (Lankry et al., 2010). The function of CD300d is related to the regulation of the expression of other CD300 molecules and the composition of CD300 complexes on the cell surface (Comas-Casellas et al., 2012).

The CD300 IR are type I TM glycoproteins with a single IgV-like extracellular domain and a membrane proximal region, a TM region, and a cytoplasmic region with ITIM motif (Lankry et al., 2010). The cytoplasmic domains of CD300a and CD300f contain internalization motifs and the cell surface expression of these molecules might be regulated, in part, by internalization (Clark et al., 2009). The expression of CD300a and CD300f was shown in MC (Clark et al., 2009), where CD300a presents on MC from nasal polyps, lungs, and on hCBMC (Bachelet et al., 2005) and its mouse ortholog LMIR-1, is expressed on mBMMC (Kumagai et al., 2003). CD300a is also expressed on NK, T cell subsets, neutrophils, eosinophils, monocytes DC, and more recently it has been characterized on basophils (Cantoni et al., 1999; Bachelet et al., 2005; Munitz et al., 2006a; Alvarez et al., 2008; Sabato et al., 2012).

It has been recently reported that CD300a interacts with polar lipids, including the major phospholipids of cell membranes, and is able to transduce intracellular signals after lipid binding. One of CD300a ligands is phosphatidylserine which directly binds CD300a and phosphorylates ITIM in the cytoplasmic portion of CD300a on BMMC (Nakahashi-Oda et al., 2012). Interestingly, Simhadri showed that CD300a phosphatidylethanolamine and phosphatidylserine bind to CD300a, and modulate the phagocytosis of dead cells. CD300a down regulates the uptake of apoptotic cells by macrophages and its ectopic expression in CD300a-negative cell lines also decreased the engulfment of dead cells (Simhadri et al., 2012). Using a co-transfection assay on THP-1 cells, Kim et al. (2012) showed that while CD300a blocked only MyD88 induced events, CD300f blocked both MyD88 and TRIF.

CD300a reduced survival of MC and eosinophils by decreasing the effect of c-kit on MC, and the IL-5, GM-CSF effect on eosinophils (Bachelet et al., 2005, 2008). Moreover a bi-specific Ab to CD300a and IgE inhibited acute airway inflammation in an experimental asthma mouse model and passive cutaneous anaphylaxis (Bachelet et al., 2008). Also a bi-specific Ab against CD300a and CCR3 inhibited chronic airway inflammation in murine asthma, possibly by its binding on both MC and eosinophils and their consequent inhibition (Munitz et al., 2006b). Disease-wise there is evidence for alterations in the expression and function of the CD300 family in patients with psoriasis (Clark et al., 2009).

Siglec-8 appears on hMCs at the same time as other MC markers such as FcεRI (Karra et al., 2009). When cross-linked by mAbs on hpbMC, Siglec-8 does not lead to apoptosis as it does on eosinophils, but rather to strong inhibition of histamine and prostaglandin D2 secretion and of $[Ca^{2+}]$ influx (Yokoi et al., 2008; Bochner, 2009; Karra et al., 2009). Siglec-8 is known to specifically recognize the sialoside sequence 6′-sulfo-sLex (Tateno et al., 2005). Siglec-8 has been shown to be associated with asthma (Gao et al., 2010). We have found *Siglec 7*, another lectin-binding IR, that was previously described on eosinophils (Munitz et al., 2006a) to be expressed and functional on hMC (Mizrahi,

S., Karra, L., Ben Zimra, M., and Levi-Schaffer, F., unpublished data).

CD72 (Lyb-2) is an ITIM-containing 45 kDa type II TM protein of the C-type lectin family (Wu and Bondada, 2009) whose natural ligands have been identified as CD100 or Semaphorin 4D (Sema4D). CD72, which is considered to be an important co-receptor regulating B-cell activation (Wu and Bondada, 2009), is also expressed on mouse NK cells (Alcon et al., 2009) and on hPBMC and human cell lines (Kataoka et al., 2010). Ligation of CD72 reduced SCF-mediated proliferation, chemotaxis, and MCP-1 (or CCL2) production in hMCs and the suppression of growth of HMC-1.2 harboring the gain-of-function mutation in KIT gene (Kataoka et al., 2010).

In conclusion IR, that have been shown to mostly modulate on MC the activation of FcεRI and c-kit, may have by themselves or by cooperation with other receptors, inhibitory functions on other receptors not necessarily linked to tyrosine kinases mechanism, although this still has to be thoroughly investigated.

CONCLUSION

Mast cells express a variety of receptors that enable them, both in activating or inhibiting ways, to modulate immune response and can be used as treatment targets in disease conditions. While with some of the described receptors such as H4 and purinergic receptors, we are close to developing a drug, with others, such as TSLP, more basic studies on their functions are needed. Overall, evidences show that MC receptors have a strong therapeutic potential but still a better understanding of their pathophysiological roles, expression and their role in the immune system is required in order to develop effective and side effect free therapeutic interventions in the near future.

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Mast cells as novel mediators of reproductive processes

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The relationship between mast cells (MCs) and pregnancy is a controversially discussed topic. The presence and quantitative distribution of MCs in the reproductive tract was confirmed in different species. A phase-dependent oscillation of MCs during the hormonal regulated estrous cycle was suggested and on this basis, MCs were assumed to play a positive role in implantation because of their ability to secrete histamine. At later pregnancy stages, they were proposed to have rather a negative role, as their exacerbated activation is associated with pre-term delivery. The present review is intended to provide an overview about uterine MCs that bring to light their unexpected relevance for reproductive processes.

Keywords: mast cells, placenta, pregnancy, uterus, implantation

INTRODUCTION

Already in the early 60s and 70s the presence and quantitative distribution of mast cells (MCs) in the reproductive tract in general and during the estrous cycle in particular was described in different species like rat (Gibbons and Chang, 1972), hamster (Harvey, 1964), and cow (Likar and Likar, 1964). The phase-dependent oscillation of MC numbers during the hormone-regulated estrous cycle was described and assumed that they are important for implantation because they secrete substances that promote tissue remodeling necessary for this process. The functional importance of these data was however unclear as the results were based on the methodological simplicity at that time. Later on, a role for MCs in reproduction was dismissed because of the apparently normal pregnancy outcome of mice lacking MCs. We have recently investigated the importance of MC deficiency in pregnancy outcome using a mouse model. The present review is intended to provide an overview about the available as well as novel data from us and many others, that bring to light the unexpected relevance of MCs for reproductive processes.

UTERINE MCs REPRESENT A DIVERGENT PHENOTYPE FROM OTHER DESCRIBED MCs

Based on their tissue specificity, murine MCs are typically classified as either mucosal or connective tissue-type MCs (Metcalfe et al., 1997). Thereby, this classification is attributed, i.a. to their histochemical staining patterns especially to those of the granule-stored proteases. Already in 1960, Spicer suggested that uterine MCs (uMCs) represent a divergent phenotype composed of mucosal as well as connective tissue-type MCs (Spicer, 1960). The histochemical observations were done by using a combined Alcian blue–Safranin stain whereby mucosal MCs are Alcian blue-positive and Safranin-negative and connective tissue-type MCs are Alcian blue-negative and Safranin-positive, respectively. In our laboratory we could confirm Spicer's findings on the presence of both MC types. uMCs were positive either for Alcian blue or for safranin. In addition, we found a third MC population that was positive for both dyes (Woidacki et al., 2013). These cells were already described and reportedly reflect different stages of differentiation (Reynolds et al., 1988; Kitamura, 1989). Moreover, they can derive from or represent an ongoing transdifferentiation process, during which mature MCs may change their content in proteoglycans, amines, peptides, etc. (Michaloudi and Papadopoulos, 1999). These conversion processes might be induced as a response to local inflammatory processes (Kitamura, 1989; Tsuji et al., 1990; van Overveld, 1990; Moon et al., 2010) or the presence of fibroblasts (Levi-Schaffer et al., 1986). Here, the most important growth factor for MCs, stem cell factor (SCF), could serve as a pivotal stimulus. It was described that MC proliferation and differentiation in the uterus is regulated by SCF secretion from uterine smooth muscle cells (Mori et al., 1997b). Furthermore, it is known that heparin is essential by controlling the levels of specific granule proteases inside MCs (Humphries et al., 1999). Thereby, the tissue-specific MC phenotype can vary in different mouse strains. In BALB/c, ear MCs express MC protease 7 (Mcpt-7), the analog of human tryptase α/β 1. In contrast, ear MCs from C57BL/6J contain no detectable protein levels of Mcpt-7 (Ghildyal et al., 1994). During pregnancy, uMCs seem to maintain their heterogeneity as in the pregnant human uterus tryptase-positive and chymase-negative (MCT) as well as tryptase-positive and chymase-positive (MCTC) MCs are present (Garfield et al., 2006). In pregnant rats, uMCs have been identified likewise as mucosal and connective tissue-type MCs based on their specific protease content (Salamonsen et al., 1996). It is therefore clear that uMCs represent a heterogeneous population of cells and they can change their phenotype according to local stimuli. Thus, uMCs constitute a special population with unique characteristics and a high plasticity, which is important to consider when designing experiments to analyze their role *in utero*.

uMCs OSCILLATE DURING THE ESTROUS CYCLE

The presence of uMCs and their menstrual or estrous cycle-related variations in number and structure were described in humans (Drudy et al., 1991a,b; Mori et al., 1997a) and other mammals like mouse (Padilla et al., 1990), rat (Aydin et al., 1998), hamster (Harvey, 1964), and goat (Karaca et al., 2008). In mouse, uMCs seem to reach their highest level during the receptive phase of the female, namely in estrus (Woidacki et al., 2013), when the uterus is prepared for nidation. This is in line with results in rats reported by Aydin et al. (1998). They detected the highest number of MCs in estrus as well. If fecundation did not occur the MC number in metestrus was decreased. After pregnancy establishment, uMC numbers became even higher (Woidacki et al., 2013). This might be due to the interplay of the sexual hormones 17β-estradiol and progesterone. In mice, maximum 17β-estradiol levels were observed at estrus whereas progesterone levels were lowest at this phase (Fata et al., 2001). Estradiol is known to potentiate the degranulation of MCs *in vitro* (Cocchiara et al., 1992). All these observations indicate a hormone-dependent regulation of uMCs. This is further reinforced by our recent observations. We found that not only MCs express the receptors for estrogens and progesterone but these hormones in combination can attract MCs *in vitro* and *in vivo* to uterine cells (Jensen et al., 2010).

MAST CELLS ARE IMPORTANT FOR INDUCTION AND MAINTENANCE OF PREGNANCY

The fundament of a successful pregnancy outcome in mammals is the maternal tolerance of the semi-allogenic fetus based on a well-orchestrated modulation of the maternal immune system and the functionality of the hormonal system. A variety of innate and adaptive immune cells are participating in this concert especially locally at the feto-maternal interface including uterine natural killer cells (Greenwood et al., 2000; Bilinski et al., 2008), dendritic cells (Blois et al., 2004; Plaks et al., 2008), and regulatory T cells (Aluvihare et al., 2004; Zenclussen et al., 2006; Schumacher, 2013), whereas the function of MCs in maternal tolerance is uncertain. High amounts of MCs were detected in the uterus during pregnancy (Menzies et al., 2012) and MC density was significantly higher in tissue from pregnant women than those of non-pregnant women (Garfield et al., 2006). We confirmed high numbers of uMCs in early pregnancy stages in a mouse model. uMCs were mainly distributed between implantation sites. Implantations from MC-deficient C57BL/6J-*Kit*$^{W-sh/W-sh}$ (W-sh), whose MC deficiency is caused by a defective *c-Kit* signaling, showed a delayed kinetic of development with a significantly diminished size in comparison to wild-type, MC sufficient controls (Woidacki et al., 2013). The transfer of bone marrow-derived MCs (BMMCs) into W-sh mice positively influenced the size of the implantation sites and restored them to normal levels (Woidacki et al., 2013). It is important to remark that a delayed implantation might have a fatal impact in pregnancy outcome (Song et al., 2002). This is further evidenced by our findings on insufficient placentation and remodeling of spiral arteries in W-sh mice (Woidacki et al., 2013). The embryo itself could act as the stimulus for the implantation process. Here, the embryo-derived histamine-releasing factor (EHRF) might be one of the first signals from the embryo to the uterus. The EHRF-induced local secretion of histamine by uMCs could play a role in preventing maternal immune rejection at the implantation stage (Cocchiara et al., 1986).

Some studies are based on histamine as an important MC-specific mediator for the initiation of blastocyst implantation processes and decidual cell responses (Shelesnyak, 1957, 1959; Nalbandov, 1971). However, the increment of the uterine histamine levels in MC-deficient WBB6F$_1$-W/W^V (W/W^V) after steroid treatment (Wordinger et al., 1985) suggests an alternative source of histamine like endothelial cells (Robinson-White et al., 1982) or/and decidual cells which have been shown to release histamine upon stimulation (Schrey et al., 1995). A study from Wordinger et al. (1986) makes the discussion even more controversial. The sterility of MC-deficient WBB6F-W/W^V is mainly due to atrophic ovaries with a hyperplastic stroma and absence of follicles and distinct corpora lutea (Wordinger et al., 1985). To determine whether implantation and live births occurred in the absence of uMCs, Wordinger et al. (1985) employed a model of ovariectomized female W/W^V mice. After the transplantation of one ovary obtained from normal female littermates (+/+) the authors transferred blastocysts from +/+ into pseudopregnant W/W^V. They could not find any differences between W/W^V and +/+ in the implantation rate after blastocyst transfer or in the number of live births. Because of these observations, Wordinger et al. (1985) excluded any requirement of uMCs in these processes. However, the transplanted ovaries were obtained from +/+ and should therefore contain a remarkable amount of already mature and differentiated MCs that could then migrate to the surrounding tissue and expand. We recently observed that locally transferred MCs into one single uterine horn were located in the other, untreated, uterine horn shortly thereafter (Woidacki et al., 2013). In the Wordinger study, no information is available regarding the presence of MCs in the ovary before and after transplantation. Additionally, the recipients were treated with steroids and MCs are known for their susceptibility to the action of hormones like estradiol and progesterone (Wordinger et al., 1985; Cocchiara et al., 1992; Rudolph et al., 2004; Jensen et al., 2010) which probably induced the expansion of the MCs present in the ovaries and their migration to the uterus. We detected high amounts of MCs within the ovaries (unpublished observations) that coincides with observations done in different species like mouse (Skalko et al., 1968), rat (Jones et al., 1980; Gaytan et al., 1991; Aydin et al., 1998; Batth and Parshad, 2000), hamster (Shinohara et al., 1987; Krishna and Terranova, 1991), cow (Reibiger and Spanel-Borowski, 2000), goat (Karaca and Simşek, 2007; Karaca et al., 2008), and chicken (Parshad and Kathpalia, 1993). Furthermore, we could not find alterations in the number of follicles as well as corpora lutea between MC-deficient W-sh and control mice (Woidacki et al., 2013). As early pregnancy is highly dependent on the presence of corpora lutea and the progesterone they secrete, we conclude that the establishment of pregnancy does not seem to depend on MCs but implantation and embryo development surely does (Woidacki et al., 2013).

On day 10 of murine normal pregnancies, MCs were present in the decidua, the maternal part of the feto-maternal interface and located closed to blood vessels (Woidacki et al., 2013). In pregnant rats, the degranulation of MCs positively influenced angiogenesis (Varayoud et al., 2004; Bosquiazzo et al., 2007).

Nevertheless, some studies exclude MCs as important mediators of pregnancy-relevant processes. Salamonsen et al. (1996) applied to syngeneically mated female rats a highly potent MC stabilizer (FPL 55618). They claimed no differences in the number of implantation sites. This is remarkable, but taking a closer look at the data, as e.g., two of a total of five rats receiving FPL intraperitoneally failed to show any embryo implantation (Salamonsen et al., 1996), meaning only 60% of the animals, got pregnant compared to more than 80% in the control group. Furthermore, the low number of animals employed it is questionable to make such strong conclusions based only in a subgroup of the studied group. Menzies et al. (2012) recently suggested that the absence of MCs had no discernible impact on pregnancy. In this study, MC-deficient C57BL/6J-*Kit*$^{W-sh/W-sh}$ and their wild-type counterparts, both syngeneically mated, had similar offspring birth weights and no difference in fetal–placental index. However, neither the kinetic nor the occurrence of implantations was analyzed or reported by these authors. That survivor animals develop normally does not discard that the first stages of pregnancy are dependent on MCs. Despite that, this study concentrated on those pregnancies that succeed after implantation and no data is provided as to how many females were pregnant after plug detection and how many blastocysts could be implanted, these authors concentrated on syngeneic matings. These two publications denying a role of MCs in pregnancy share one aspect: they are based on syngeneic and therefore biologically questionable matings. Naturally occurring pregnancies *in natura* are predominantly allogeneic to maintain the genetical variability of a species. That allows the adaptation of the fetus to its later environment at the best. Matings with genetically related and even worse among identic individuals has to be avoided because of the partially tremendous consequences of inbreeding. Syngeneic matings are exclusively necessary to maintain inbreeding colonies in the laboratories. Even there, after some generations mostly a backcross to wild-types has to be done as a result of the genetic impoverishment. Madeja et al. (2011) could show that murine allogeneic fetuses and placentas were heavier at term compared with syngeneic controls. This consequence was based on impaired decidual vascularization as well as placental and fetal growth after syngeneic matings. They supposed that allogeneic placentas are much more sufficient in supporting fetal growth by adequate modulation of spiral arteries. It seems reasonable to assume that paternal allo-antigens are important for stimulating maternal immune cells, which is not further discussed as it would go beyond the scope of this article. In this context, the role of MCs is worth to be studied and critically analyzed in efficient, relevant allogeneic pairings as the results obtained in syngeneic ones are limited by the already mentioned factors.

MC-deficient C57BL/6J-*Kit*$^{W-sh/W-sh}$ (W-sh) mice implanted significantly less blastocysts than their wild-type counterparts after allogeneic mating. Uteri from W-sh mice were either very thick, swollen, and reddish with no visible implantations or contained few implantations. Accordingly, their litter size was significantly reduced as compared to wild-type controls. The systemic and local reconstitution with BMMC completely restored the reproductive phenotype of W-sh mice. Moreover, the few implanted blastocysts in W-sh mice developed significantly smaller placentas and insufficient modifications of the spiral arteries that are responsible for supplying oxygen and nutrients to the fetus. BMMC transfer normalized all parameters and therefore contributed to a normal pregnancy outcome by mediating placental development and spiral artery remodeling (Woidacki et al., 2013).

Inadequate placental development mainly due to discrepancies in trophoblast differentiation and invasion, respectively can lead to intrauterine growth retardation (IUGR) and pre-eclampsia amongst other complications. In placentas obtained from IUGR-pregnancies the number of MCs was markedly decreased while hypoxia could intensify MC degranulation (Szukiewicz et al., 1999b). The degranulation of MCs resulted in a greater increase of the vascular resistance in pre-eclampsia likely due to the vasoconstrictive function of histamine (Szukiewicz et al., 1999a) and asthmatic pregnant women are at increased risk to develop this disease (Siddiqui et al., 2008; Murphy et al., 2011). In severe pre-eclampsia, the number of human MC chymase-positive cells was significantly higher compared to normal pregnant women (Mitani et al., 2002) and MC-chymase is known to be more potent than angiotensin-converting enzyme to convert angiotensin I to angiotensin II (Wintroub et al., 1984). Whether the secreted histamine, that inhibits the apoptotic activity in trophoblast cells via H(1) receptor (Pyzlak et al., 2010) and further influences the process of trophoblast differentiation (Szewczyk et al., 2005) and invasion (Liu et al., 2004) is derived exclusively from MCs has to be still determined. Thus, the heterogenicity of uMCs is also depicted here: low numbers of uMCs is associated with pathologies as IUGR which would predict a positive role of uMCs on fetal growth while their exacerbated activation is related to pre-eclampsia and pre-term birth. Hence, uMCs represent a heterogenous population, which shows also a high plasticity to respond differently to different stimuli.

MCs AND THEIR INFLUENCE ON PERINATAL PROCESSES

There are strong hints for the relevance of MCs in mediating the implantation of the blastocyst as discussed above. As pregnancy advances, MCs exert an influence on the maintenance of pregnancy by allowing the unrestricted development of the placenta and remodeling of the spiral arteries (Woidacki et al., 2013). Interestingly, there are vast evidences that MCs also influence perinatal processes. The degranulation of MCs can lead to substantial changes in the myometrial contractility (Martínez et al., 1999; Garfield et al., 2006). Resident MCs increased uterine contractility in pregnant guinea pigs through multiple mediators including histamine and serotonin. Uterine responses to these mediators are dependent on gestational age (Bytautiene et al., 2008). Pregnant women affected by systemic mastocytosis exhibit manifestations of pre-term labor and delivery. This disease is accompanied by an unexplained and pathologic increase in MCs in specific tissue (Metcalfe and Akin, 2001). The allergic activation of MCs results in a substantial increase in uterine contractility (Garfield et al., 2006) and could be therefore responsible for the allergy-associated induction of pre-term labor (Habek et al., 2000; Bytautiene et al., 2004). This is in line with the fact that pregnant women with asthma are at a higher risk to pre-term delivery (Perlow et al., 1992; Sorensen et al., 2003; Murphy et al.,

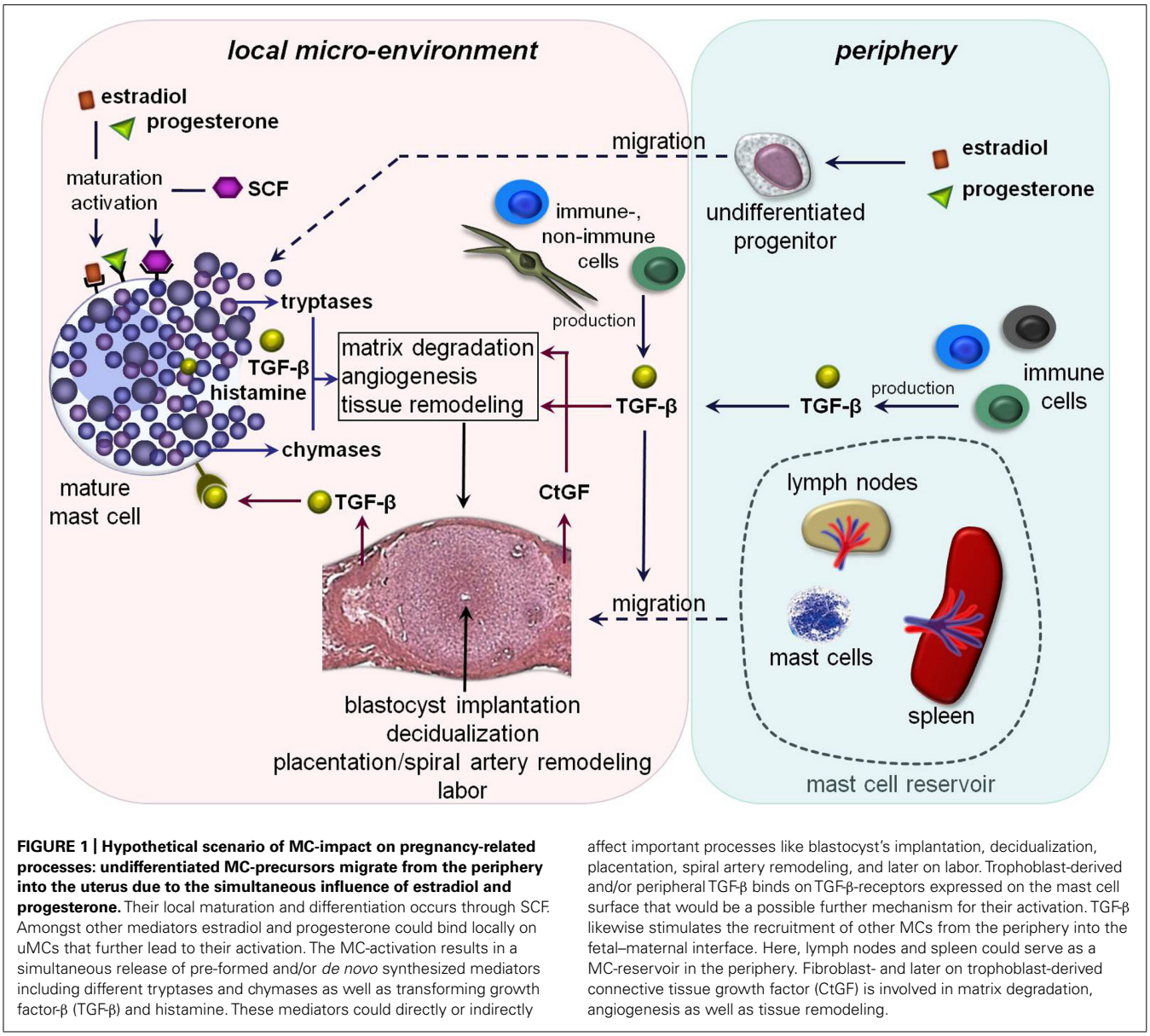

FIGURE 1 | Hypothetical scenario of MC-impact on pregnancy-related processes: undifferentiated MC-precursors migrate from the periphery into the uterus due to the simultaneous influence of estradiol and progesterone. Their local maturation and differentiation occurs through SCF. Amongst other mediators estradiol and progesterone could bind locally on uMCs that further lead to their activation. The MC-activation results in a simultaneous release of pre-formed and/or *de novo* synthesized mediators including different tryptases and chymases as well as transforming growth factor-β (TGF-β) and histamine. These mediators could directly or indirectly affect important processes like blastocyst's implantation, decidualization, placentation, spiral artery remodeling, and later on labor. Trophoblast-derived and/or peripheral TGF-β binds on TGF-β-receptors expressed on the mast cell surface that would be a possible further mechanism for their activation. TGF-β likewise stimulates the recruitment of other MCs from the periphery into the fetal–maternal interface. Here, lymph nodes and spleen could serve as a MC-reservoir in the periphery. Fibroblast- and later on trophoblast-derived connective tissue growth factor (CtGF) is involved in matrix degradation, angiogenesis as well as tissue remodeling.

2006). However, Menzies et al. (2012) concluded that MCs have no impact on initiation of labor because the time of labor initiation in MC-deficient mice was indistinguishable from wild-type controls. Nevertheless, the number of MCs within the mouse cervix doubled from non-pregnant to day 18 of pregnancy, with a further 1.5-fold increase with labor (Menzies et al., 2012). This relevant question is worth to be tested in a mouse model for pre-term delivery and remains highly up-to-date.

In summary our data as well as data from the literature show that MCs accompany and deeply affect many steps of reproduction. MCs are modified and attracted by hormones, uMCs are essential for allowing implantation of allogeneic embryos, and positively influence placentation and thus, embryo development. Later on, an exacerbated number or function of uMCs can negatively influence pregnancy and foster pre-term delivery. It is clear that uMCs are not only different from other MCs because of their unique markers but also seem to secrete different mediators at different pregnancy stages and upon different stimuli. This makes these cells an extremely interesting target of study for both, reproductive biologists and MC researchers. Based on the data discussed in this review, we propose following hypothetical scenario for the impact of MCs on pregnancy-related processes.

CONCLUSION

Mast cells vitally influence reproductive processes and in particular the pregnancy itself by modulating non-immunological responses like tissue remodeling, angiogenesis, optimal placentation, and spiral artery modifications as well as labor. They further play a rather negative role in parturition as the excessive secretion of MC-mediators may lead to pre-term delivery. MCs may act not only as mediators of the innate immune system but also as cellular switch points between innate and adaptive immune responses.

Their activity is regulated by endocrine and physiological signals and based on their granule-stored array of biologically active products. All these well-orchestrated mechanisms allow the non-restrictive development of the semi-allogeneic fetus within the maternal uterus and therefore fetal survival. The understanding of the paradoxon "pregnancy" is of fundamental importance for helping couples to realize their often unfulfilled desire to have children. In this context, the data in regard to the mast cell-associated positive pregnancy outcome might serve as a further puzzle piece to answer these questions.

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