Integrated signalling pathways for mast-cell activation

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Abstract | Mast-cell activation mediated by the high-affinity receptor for IgE (FcεRI) is considered to be a key event in the allergic inflammatory response. However, in a physiological setting, other receptors, such as KIT, might also markedly influence the release of mediators by mast cells. Recent studies have provided evidence that FcεRI-dependent degranulation is regulated by two complementary signalling pathways, one of which activates phospholipase Cγ and the other of which activates phosphatidylinositol 3-kinase, using specific transmembrane and cytosolic adaptor molecules. In this Review, we discuss the evidence for these interacting pathways and describe how the capacity of KIT, and other receptors, to influence FcεRI-dependent mast-cell-mediator release might be a function of the relative abilities of these receptors to activate these alternative pathways.

Pathogen-associated molecular patterns
(PAMPs). Molecular motifs that are characteristic of prokaryotes and are thereby recognized by the mammalian innate immune system.

Eicosanoids
A family of lipid mediators with diverse biological activities. These metabolites are the products of the lipoxygenase and cyclooxygenase pathways — which produce leukotrienes and prostaglandins, respectively — and they are important mediators of the allergic inflammatory response.

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Mast cells are derived from $CD13^{+}CD34^{+}CD117^{+}$ (also known as KIT) haematopoietic progenitors in the bone marrow[1]. Developing mast cells subsequently migrate to peripheral tissues, such as the skin, mucosa and airways, where they terminate their differentiation under the influence of factors in the tissue micro-environment[2] and participate in the regulation of adaptive immune responses[3,4]. These cells are also now thought to contribute to host-defence mechanisms that are associated with innate immunity[5]. Studies of mast-cell signal transduction, however, have mainly been driven by the acknowledged central role of these cells in allergic inflammatory responses[1]. 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 IgE-bound high-affinity receptors for IgE (FcεRIs) expressed at the mast-cell surface[6] (FIG. 1). There is an increasing realization, however, that receptors for other ligands — such as adenosine, complement component 3a (C3a), chemokines, cytokines, pathogen-associated molecular patterns (PAMPs), sphingosine 1-phosphate (S1P) and stem-cell factor (SCF; also known as KIT ligand) — might markedly influence mast-cell activation in a physiological setting (FIG. 1; TABLE 1). These receptors — which can either potentiate FcεRI-mediated mast-cell activation or, by themselves, stimulate the release of mast-cell mediators — initiate their signalling cascades by different mechanisms. Ultimately, however, these cascades must converge to allow the necessary downstream signalling events that are required for degranulation, release of eicosanoids, and induction of expression of chemokines and cytokines.

In this Review, we describe the signalling events that are crucial for mast-cell-mediator release, discuss the evidence for integrated 'principle' initiating and 'complementary' (amplification) signalling pathways for the regulation of mast-cell activation, and describe how other receptors that are expressed by mast cells might use these alternative pathways to regulate FcεRI-dependent responses.

FcεRI-mediated mast-cell activation

Antigen-dependent mast-cell activation is regulated by a complex series of intracellular signalling processes that is initiated following FcεRI aggregation. Although the immediate receptor-proximal signalling events seem to be common for the release of all categories of mediator, the receptor-distal signalling events must diverge to regulate the different mechanisms by which these mediators are released (FIG. 1).

FcεRI is a tetrameric receptor that comprises an α-chain, which is responsible for binding IgE, as well as a β-chain and a disulphide-linked γ-chain homodimer, which are responsible for initiating signalling. The immediate receptor-proximal events that lead to activation of downstream signalling molecules are still not fully understood. However, as for other members of the immunoglobulin-receptor superfamily, such as the B-cell receptor[7] and T-cell receptor[8], it is clear that these initial signalling events involve coalescence of the aggregated

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Lipid rafts
This term conceptually describes detergent-resistant membrane fractions that are separated on sucrose gradients. These fractions contain heterogeneous plasma-membrane microdomains that are enriched in sphingolipids, cholesterol and glycosylphosphatidylinositol-anchored proteins, as well as several membrane-associated signalling molecules, such as LYN. These microdomains are thought to be important sites for protein-tyrosine-kinase-mediated protein–protein interactions that are involved in the initiation of receptor signalling pathways.

receptors with specialized microdomains of the plasma membrane known as lipid rafts[9], activation of SRC-family kinases[10] and, subsequently, tyrosine phosphorylation of the receptor subunits[11,12]. In mast cells, the main SRC-family kinase that is involved in these initial stages is LYN, which mainly resides in lipid rafts[13]. The term lipid raft conceptually defines detergent-resistant membrane fractions that are isolated on sucrose gradients. The components of these fractions are likely to be heterogeneous, originating from multiple locations within the plasma membrane[14]. Nevertheless, it has been documented that the activity of LYN present in lipid rafts is considerably higher than that of LYN outside these microdomains, owing to the exclusion of inactivating phosphatases from these regions[15]. So, the association of aggregated FcεRI with the preferentially activated LYN in lipid rafts might be sufficient to shift the equilibrium of FcεRI from a non-phosphorylated state to a phosphorylated state, thereby initiating FcεRI-mediated degranulation (FIG. 2).

There has been confusion, however, regarding the exact requirement for LYN in mast-cell activation. Much of this confusion has arisen from the different phenotypes observed for *Lyn*$^{-/-}$ mice and mast cells derived from the bone marrow (BMMCs) of these mice (TABLE 2). In this respect, it was reported that antigen-mediated allergic reactions were absent in *Lyn*$^{-/-}$ mice[16]. However, a later report indicated that, at least in young *Lyn*$^{-/-}$ mice, passive-cutaneous-anaphylaxis reactions and/or passive-systemic-anaphylaxis reactions were of increased intensity in the absence of LYN[17]. Furthermore, various phenotypes have been described for *Lyn*$^{-/-}$ BMMCs: one phenotype shows FcεRI-mediated degranulation at wild-type levels[18]; a second shows less FcεRI-mediated degranulation than wild-type BMMCs[19]; and a third shows more FcεRI-mediated degranulation than wild-type BMMCs[17,20,21]. The reasons for these contradictory data remain unclear; however, possible explanations for these disparities include back-crossing of the original C57BL/6 mice to C57BL/6 × 129/Sv mice, different ages of the mice and/or different culture conditions for the BMMCs. A further possibility is that LYN regulates both positive pathways and negative pathways for FcεRI-mediated degranulation, the latter through the phosphatase SHIP (SRC homology 2 (SH2)-domain-containing inositol-5-phosphatase)[21], and that observed responses reflect alterations in the balance between these two phenomena under varied experimental conditions. Nevertheless, a common feature to all of the *Lyn*$^{-/-}$ BMMC phenotypes is the occurrence of residual degranulation in response to antigen, despite marked impairment of antigen-mediated FcεRI β- and γ-chain tyrosine phosphorylation and defective downstream signalling[18–21] (TABLE 2). These observations imply that other signalling pathways, which are independent of LYN, might be involved in the regulation of FcεRI-mediated mast-cell activation; this concept is discussed later.

The tyrosine residues that are phosphorylated by LYN in the FcεRI β-chain and γ-chain are present in the immunoreceptor tyrosine-based activation motifs (ITAMs). When phosphorylated, the β- and γ-chain ITAMs provide high-affinity docking sites for the SH2 domains of LYN[22] and for the SH2 domains of the ZAP70 (ζ-chain-associated protein kinase of 70 kDa)-related tyrosine kinase SYK (spleen tyrosine kinase)[23], respectively. The tethering of SYK in this manner allows *trans*- and autophosphorylation of its catalytic domain, as well as phosphorylation by LYN, thereby increasing its catalytic activity[24,25]. The subsequent SYK- and/or LYN-mediated tyrosine phosphorylation of the transmembrane adaptor molecule LAT (linker for activation of T cells) is crucial for coordination of the downstream signalling pathways that are required for the release of the various pro-inflammatory mediators[26] (FIG. 2; TABLE 2).

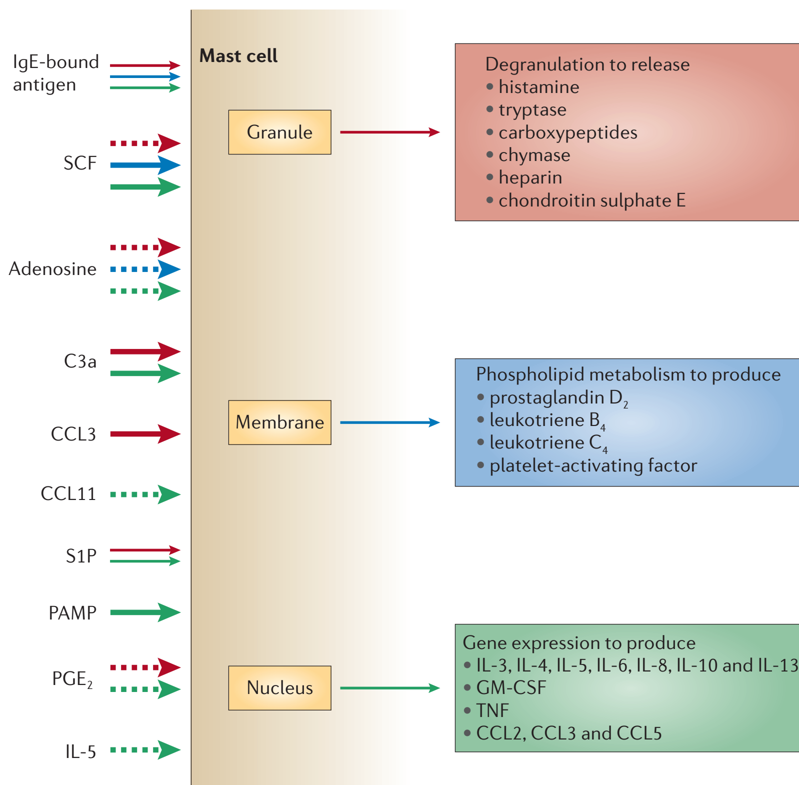

Figure 1 | **Influence of environmental and/or physiological stimuli on the release of pro-inflammatory mediators by mast cells.** The colour of the arrows denotes the category of mediator that is released. The solid arrows signify that the agent induces mediator release on its own, whereas the dashed arrows signify that the agent can induce mediator release only in the presence of antigen. The thick arrows signify that the agent increases antigen-mediated responses. C3a, complement component 3a; CCL, CC-chemokine ligand; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; PAMP, pathogen-associated molecular pattern; PGE_2, prostaglandin E_2; S1P, sphingosine 1-phosphate; SCF, stem-cell factor; TNF, tumour-necrosis factor.

LAT and NTAL link to GADS and/or GRB2

LAT was originally identified in the laboratory of Lawrence Samelson as a 36–38-kDa substrate for ZAP70 in activated T cells[27]. Owing to its juxta-membrane palmitoylation site, LAT resides mainly in lipid rafts; however, the ability of LAT to associate with lipid rafts does not seem to be required for its function[28]. As shown in T cells and/or mast cells[26,27,29–31],

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Table 1 | **Ligands and their receptors that influence mast-cell activation**

Receptor	Species	Ligand	Source of ligand	Initiating signals	Mast-cell response	Refs
Fc						
FcεRI, FcγRI, FcγRIII	Mouse, rat, human	IgE-bound antigen	B cells	SRC-family kinases and SYK	• Induced degranulation • Induced eicosanoid, cytokine and chemokine release	106–111
G-protein coupled						
C3aR	Human	C3a	Hepatocytes	$G_{\alpha i}$	• Induced degranulation and chemokine production • Increased FcγRI-dependent degranulation	101,102
A_3R	Mouse	Adenosine	Mast cells, pro-inflammatory cells	$G_{\alpha i}$	• Increased and sustained FcεRI-dependent degranulation	89
CCR1	Mouse	CCL3	Mast cells	$G_{\alpha i}$	• Induced degranulation • Increased FcεRI-dependent degranulation	98
CCR3	Human	CCL11	Epithelial cells, pro-inflammatory cells	$G_{\alpha i}$	• Increased FcεRI-dependent IL-13 secretion	112
$S1P_2$	Mouse	S1P	Mast cells	$G_{\alpha i}$	• Induced degranulation	78
Cytokine						
IL-3R	Human	IL-3	Mast cells, T cells	JAK–STAT	• Increased FcεRI-dependent histamine and LTC_4 release	113
IL-4R	Mouse	IL-4	Mast cells, T cells	JAK–STAT	• Inhibited FcεRI-dependent cytokine release • Increased FcγR-dependent degranulation and cytokine production • Inhibited FcεRIβ expression and increased FcγRIIIα expression	114,115
	Human	IL-4	Mast cells, T cells	JAK–STAT	• Increased FcεRI-dependent histamine, LTC_4 and IL-5 release	116,117
IL-5R	Human	IL-5	Mast cells, T cells	JAK–STAT	• No effect on FcεRI-dependent degranulation • Increased FcεRI-dependent cytokine release	117
IL-10R	Mouse, rat, human	IL-10	Mast cells, T cells	JAK–STAT	• No effect on FcεRI-dependent degranulation • Decreased FcεRI-dependent cytokine release • Decreased FcεRIβ expression	118–120
TGFβR1	Mouse, rat	TGFβ	Mast cells, pro-inflammatory cells	JAK–STAT	• Decreased FcεRI-dependent degranulation • Decreased FcεRI-dependent TNF production • Decreased FcεRIα and FcεRIβ expression	121,122
KIT	Mouse, rat, human	SCF	Fibroblasts, endothelial cells, bone-marrow cells, stromal cells	KIT, SRC-family kinases and PI3K	• Induced cytokine and chemokine release • Induced growth, differentiation and adhesion • Increased FcεRI-dependent degranulation • Increased FcεRI-dependent cytokine release	36,91–94,97
Other						
TLRs	Mouse, rat, human	PAMP	Bacteria, viruses	MyD88, IRAK and TRAF	• Induced degranulation • Induced eicosanoid and cytokine release	5

A_3R, A_3 adenosine receptor; C3a, complement component 3a; C3aR, C3a receptor; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; FcγRI, high-affinity receptor for IgG; FcεRI, high-affinity receptor for IgE; FcγRIII, low-affinity receptor for IgG; $G_{\alpha i}$, α-subunit of inhibitory G protein; IL, interleukin; IL-3R, IL-3 receptor; IL-4R, IL-4 receptor; IL-5R, IL-5 receptor; IL-10R, IL-10 receptor; IRAK, IL-1-receptor-associated kinase; JAK, Janus kinase; LTC_4, leukotriene C_4; MyD88, myeloid differentiation primary-response protein 88; PAMP, pathogen-associated molecular pattern; PI3K, phosphatidylinositol 3-kinase; S1P, sphingosine 1-phosphate; $S1P_2$, S1P receptor 2; SCF, stem-cell factor; STAT, signal transducer and activator of transcription; SYK, spleen tyrosine kinase; TGFβ, transforming growth factor-β; TGFβR1, TGFβ receptor 1; TLR, Toll-like receptor; TNF, tumour-necrosis factor; TRAF, TNF-receptor-associated factor.

phosphorylation of LAT results in the recruitment of several types of molecule: cytosolic adaptor molecules, such as GRB2 (growth-factor-receptor-bound protein 2), GADS (GRB2-related adaptor protein), SHC (SH2-domain-containing transforming protein C) and SLP76 (SH2-domain-containing leukocyte protein of 65 kDa); guanine-nucleotide-exchange factors and adaptor molecules, such as SOS (son of sevenless homologue) and VAV; and signalling enzymes, such as phospholipase $C\gamma_1$ ($PLC\gamma_1$) (FIG. 2). These interactions with LAT — which take place either directly (as for GADS, GRB2 and $PLC\gamma_1$) or indirectly (as for SOS, SLP76 and SHC, which bind LAT indirectly through GRB2, GADS and GRB2, respectively[29]) — result in the formation of a macromolecular signalling complex, which allows the diversification of downstream signalling that is required for the release of the various pro-inflammatory mediators. Studies carried out in

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SRC-family kinases
A family of closely related protein tyrosine kinases — including SRC, LYN, LCK, FYN and BLK (B-lymphoid kinase) — each member of which has a SRC homology 2 (SH2) domain, an SH3 domain, a single catalytic domain, a carboxy-terminal regulatory domain and a unique amino-terminal region. These proteins are anchored at the plasma membrane and targeted to lipid rafts as a result of myristoylation and palmitoylation.

LAT-deficient mice indicate that LAT is indispensable for the development of T cells[32] and for T-cell-receptor-ligation-induced signalling events, such as calcium mobilization[31]. Similarly, BMMCs from these mice have a marked impairment in FcεRI-mediated degranulation and cytokine production[26]. However, unlike the absolute requirement for LAT in T-cell responses, residual FcεRI-mediated calcium mobilization, degranulation and cytokine production is still observed in LAT-deficient BMMCs[26], implying the presence of a functional transmembrane adaptor molecule in these cells that is distinct from LAT.

A candidate for this function was isolated in the laboratories of Václav Hořejší[33] and Weiguo Zhang[34]. Their research groups independently identified a novel transmembrane adaptor molecule, which was named NTAL (non-T-cell activation linker)[33] and LAB (linker for activation of B cells)[34], respectively. This molecule is structurally similar to LAT[33,34] and, as such, has now been renamed LAT2 by The Human Genome Organisation (HUGO) Gene Nomenclature Committee. In this Review, however, we use the term NTAL, because this reflects the more commonly used terminology. Unlike LAT, NTAL is not expressed by resting T cells but, instead, by B cells, natural killer cells and, as for LAT, mast cells[33,34]. Similar to LAT, NTAL contains a palmitoylation site for targeting to lipid rafts; however, NTAL seems to be localized in different membrane microdomains than LAT[35]. Multiple tyrosine residues in the cytoplasmic tail of NTAL also provide potential docking sites for molecules that can associate following phosphorylation[33,34]. A role for NTAL in mast-cell function was indicated by its rapid LYN- and SYK-dependent tyrosine phosphorylation in both mouse[33,36] and human[36] mast cells following FcεRI aggregation, as well as by the ability of small interfering RNA (siRNA) knockdown of *NTAL* mRNA to reduce FcεRI-dependent degranulation by human mast cells[36]. Subsequent studies showed that the residual antigen-mediated calcium mobilization and degranulation observed for LAT-deficient BMMCs was almost abolished in BMMCs that were deficient in both LAT and NTAL, indicating that NTAL functions in a complementary manner (together with LAT) to regulate degranulation[35,37]. *Ntal*$^{-/-}$ BMMCs, however, have a higher capacity to signal and to degranulate and generate specific cytokines in response to antigen than do wild-type BMMCs[35,37], indicating that NTAL might also be a negative regulator of FcεRI-mediated mast-cell activation[35,37]. This negative regulation has been proposed to be a consequence of competition between NTAL and LAT for the same pool of associating signalling molecules in lipid rafts[35]. However, challenge of *Ntal*$^{-/-}$ BMMCs with antigen results in increased LAT phosphorylation and LAT-dependent signalling events[35,37], so another explanation could be that LAT is overcompensating for the defects in the NTAL-regulated signalling pathway in these cells. For further discussion of the relative roles of LAT and NTAL in mast-cell activation, see REF. 38.

Similar to LAT, phosphorylated NTAL binds GRB2; therefore, there is likely to be a degree of overlap and redundancy in the function of these two molecules[33,34]. In B cells, GRB2 seems to have a negative-regulatory role in antigen-mediated calcium mobilization and cell activation, and this role is eliminated after GRB2 binds NTAL[39]. So, it has been proposed that NTAL functions as an amplifier of antigen-mediated B-cell activation by switching off inhibitory responses[39]. Whether a similar mechanism operates in mast cells is unknown. Examination of the amino-acid sequences of LAT and NTAL (FIG. 3) shows that both LAT and NTAL contain five potential GRB2-binding sites (YXN, where X denotes any amino acid). However, at least for LAT, only the three terminal GRB2-binding sites (Y171, Y191 and Y226) seem to be phosphorylated and required for LAT function in mast cells. In addition to the GRB2-binding sites, other recognized binding sites are found in both

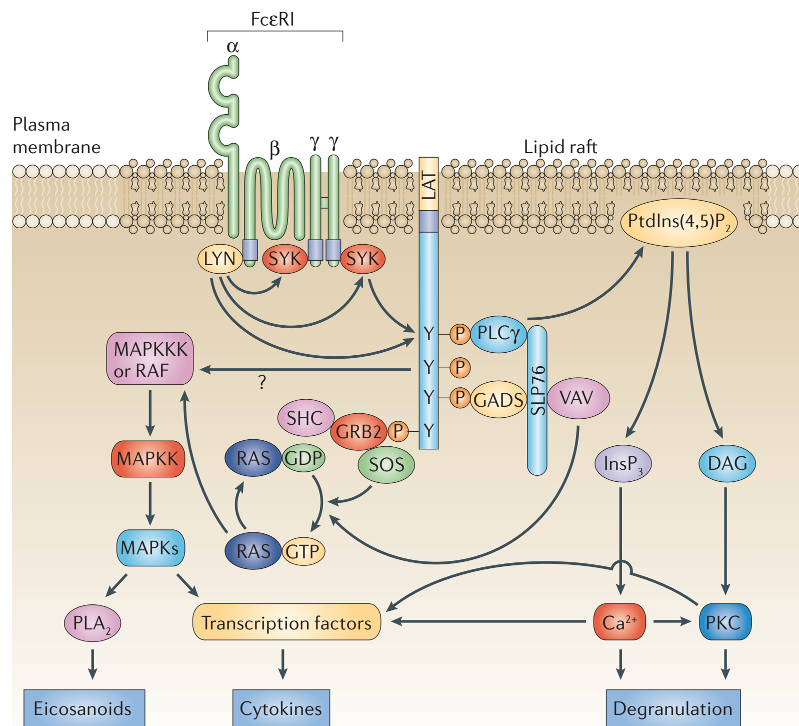

Figure 2 | **The 'principle' signalling cascade in activated mast cells.** For clarity, only one high-affinity receptor for IgE (FcεRI) is shown. Following FcεRI aggregation, the adaptor molecule LAT (linker for activation of T cells) becomes phosphorylated in a LYN- and SYK (spleen tyrosine kinase)-dependent manner. This results in direct or indirect binding to LAT of the cytosolic adaptor molecules GRB2 (growth-factor-receptor-bound protein 2), GADS (GRB2-related adaptor protein), SHC (SRC homology 2 (SH2)-domain-containing transforming protein C) and SLP76 (SH2-domain-containing leukocyte protein of 76 kDa), as well as the exchange factors and adaptor molecules VAV and SOS (son of sevenless homologue), and the signalling enzyme phospholipase Cγ$_1$ (PLCγ$_1$). Again, for clarity, only one binding site is depicted for GRB2 and GADS. Degranulation follows the activation of PLCγ and protein kinase C (PKC) and the increased mobilization of calcium (Ca^{2+}), whereas the generation of eicosanoids (including leukotriene C$_4$ and prostaglandin D$_2$) and cytokines follows the activation of the RAS–RAF–mitogen-activated protein kinase (MAPK) pathway. Although the cascade that leads to activation of the MAPKs extracellular-signal-regulated kinase 1 (ERK1) and ERK2 is known to be regulated by RAF, the pathways that regulate the MAPK kinases (MAPKKs) and the MAPKK kinases (MAPKKKs) that mediate p38 and JUN amino-terminal kinase (JNK) activation in mast cells are less well-defined. DAG, diacylglycerol; InsP$_3$, inositol-1,4,5-trisphosphate; PLA$_2$, phospholipase A$_2$; PtdIns(4,5)P$_2$, phosphatidylinositol-4,5-bisphosphate.

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Passive cutaneous anaphylaxis
An experimental technique that reflects *in situ* mast-cell degranulation. Mice or rats are passively sensitized by intradermal injection of antigen-specific IgE, then antigen and the dye Evans Blue are injected into the tail vein. Mediators that are released from the activated mast cells increase vascular permeability, resulting in leakage of the dye into the skin at the site of injection of IgE.

LAT and NTAL, but these sites are not common to both molecules. In this respect, LAT, but not NTAL, has a site (Y132) that, when phosphorylated, allows binding of the SH2 domain of PLCγ_1. In addition, binding sites for GADS that are present in phosphorylated LAT, but not NTAL, have been mapped to Y171 and Y191, both of which also bind GRB2 (as described earlier). NTAL, but not LAT, has a region between residues 104 and 114 that contains the sequence YIDP (FIG. 3b), which is highly homologous to a region in KIT that binds SRC-family kinases, such as LYN, FYN and the protein phosphatase SHP1 (SH2-domain-containing protein tyrosine phosphatase 1)[40]. NTAL also contains three putative SH3-domain recognition motifs (RXXK), which are also not found in LAT. In SLP76, this sequence (RXXK) has been shown to confer binding to the SH3 domain of GADS[41], and in the immune-cell adaptor protein SKAP55 (SRC-kinase-associated phosphoprotein of 55 kDa), it confers binding to the SH3 domain of FYN[42,43]. However, such interactions of GADS and/or FYN with NTAL, which might explain how NTAL positively regulates FcεRI-mediated mast-cell degranulation, have yet to be shown. Similarly, binding of SHP1 or other phosphatases to NTAL, which could explain the increased FcεRI-dependent responses in *Ntal*$^{-/-}$ BMMCs, has also not been reported.

LAT-regulated PLCγ activation

The four terminal tyrosine residues in LAT (Y132, Y171, Y191 and Y226) are crucial and sufficient for the ability of LAT to regulate signalling in, and degranulation by, mast cells[44]. The main signalling enzyme, which is regulated by both direct and indirect interactions with these tyrosine residues, is PLCγ. When activated, PLCγ catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) in the plasma membrane.

Table 2 | **The effects on mast-cell function of gene knockout or knockdown of key signalling molecules**

Gene knockout, knockdown or mutation	Effect on degranulation	Effect on cytokine release	Effect on signalling	References
Lyn knockout	Decreased	Increased	• Decreased FcεRIβ and FcεRIγ phosphorylation • Prolonged MAPK and JUN phosphorylation	19
	Increased	ND	• Increased PI3K activity, owing to GAB2 phosphorylation • Increased PI3K activity, owing to decreased SHIP activity • Compensation by FYN	17,20,21
	No change	ND	• Impaired tyrosine phosphorylation and decreased Ca^{2+} signal	18
Fyn knockout	Decreased	Decreased	• Decreased PI3K activity • No change in Ca^{2+} signal	20
Syk knockout	Abolished	Abolished	ND	123
Vav knockout	Decreased	Decreased	• Decreased PLCγ-mediated Ca^{2+} signal and decreased PI3K activity	56
Slp76 knockout	Decreased	Decreased	• Decreased PLCγ_1 phosphorylation and decreased Ca^{2+} signal	55
Gab2 knockout	Decreased	Decreased *Il6* mRNA synthesis	• Blocked PI3K-dependent pathway	71
Lat knockout	Decreased	Decreased	• Decreased SLP76, PLCγ_1 and PLCγ_2 phosphorylation • Decreased MAPK activity and decreased Ca^{2+} signal • No change in SYK and VAV phosphorylation	26
Lat knockdown	Decreased	ND	ND	36
Ntal knockout	Increased (no change in *in vivo* anaphylaxis)	Increased	• Hyperphosphorylation of LAT, PLCγ_1, ERK1 and ERK2	35,37
Ntal knockdown	Decreased	ND	ND	36
Ntal and *Lat* knockout	Decreased*	Decreased*	• Blocked LAT and PLCγ phosphorylation and blocked Ca^{2+} signal	35,37
Btk knockout	Decreased	Decreased	• Decreased $InsP_3$ production and decreased Ca^{2+} signal	19,124
Btk and *Lyn* knockout	Decreased	Decreased	• Decreased PLCγ-, ERK- and JUN-signalling pathways	19
p85a (PI3K subunit) knockout	No change	ND	• Blocked PI3K activation	50,125
p110d (PI3K subunit) mutant	Decreased (blocked KIT-mediated responses)	Decreased	• Blocked PI3K activation	75

*Less than released by *Lat*-knockout bone-marrow-derived mast cells. *Btk*, Bruton's tyrosine kinase gene; Ca^{2+}, calcium ion; ERK, extracellular-signal-regulated kinase; FcεRI, high-affinity receptor for IgE; GAB2, growth-factor-receptor-bound protein 2 (GRB2)-associated binding protein 2; *Il6*, interleukin-6 gene; $InsP_3$, inositol-1,4,5-trisphosphate; LAT, linker for activation of T cells; MAPK, mitogen-activated protein kinase; ND, not determined; *Ntal*, non-T-cell activation linker gene; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase Cγ; SCF, stem-cell factor; SHIP, SRC-homology-2-domain-containing inositol-5-phosphatase; SHP2, SRC-homology-2-domain-containing protein tyrosine phosphatase 2; SLP76, SRC-homology-2-domain-containing leukocyte protein of 76 kDa; SYK, spleen tyrosine kinase.

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The resulting products, inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol[45,46], induce mobilization of cytosolic calcium and activation of protein kinase C (PKC), respectively. The calcium signal induced by $InsP_3$ is transient; however, calcium sequestered from extracellular stores by capacitive entry, as a consequence of depletion of intracellular stores[47], allows this signal to be sustained. Two structurally similar isoforms of PLCγ are expressed by mast cells: $PLC\gamma_1$ and $PLC\gamma_2$ (REF. 48). Both isoforms require plasma-membrane localization and subsequent phosphorylation for their activation[48]. As monitored by these parameters, both $PLC\gamma_1$ and $PLC\gamma_2$ are activated following FcεRI aggregation at the surface of mouse BMMCs[26] and cells of the rat mast-cell line RBL-2H3 (rat basophilic leukaemia 2H3)[49]. In human mast cells, however, despite membrane translocation of both isoforms and marked phosphorylation of $PLC\gamma_1$, following antigen challenge, phosphorylation of $PLC\gamma_2$ has been difficult to detect[50], indicating that $PLC\gamma_1$ might be the main active form (at least in human mast cells).

Because knockout of the gene that encodes $PLC\gamma_1$ is developmentally lethal[51], the relative contributions of $PLC\gamma_1$ and $PLC\gamma_2$ to mast-cell activation have been difficult to assess. However, BMMCs from $PLC\gamma_2$-deficent mice have a reduced capacity to degranulate[52]. Furthermore, preliminary studies have shown that the PLCγ inhibitor U73122 completely inhibits FcεRI-mediated calcium mobilization in, and degranulation by, human mast cells (C.T., A.M.G. and M. A. Beaven, unpublished observations). So, the PLCγ-dependent increases in both cytosolic free calcium and PKC activation are essential signals for degranulation to proceed[53,54].

FcεRI-dependent phosphorylation of $PLC\gamma_1$ and $PLC\gamma_2$ is almost abolished and the calcium signal is reduced in $Lat^{-/-}$ BMMCs[26]. Not only is the Y132 $PLC\gamma_1$-binding site in LAT crucial for this ability of LAT to regulate $PLC\gamma_1$ but, as shown using the Jurkat T-cell line, the terminal tyrosine residues that are responsible for binding GRB2 and/or GADS are also essential[31]. These findings, as well as other observations[31], have led to the conclusion that the indirect binding of $PLC\gamma_1$ (through its interaction with GADS-linked SLP76), in addition to the direct binding of $PLC\gamma_1$ to LAT, is essential for its localization at the plasma membrane. The observation that antigen-stimulated $Slp76^{-/-}$ BMMCs[55] have a similar phenotype to $Lat^{-/-}$ BMMCs supports this conclusion. The studies discussed here therefore allow us to conclude that LAT regulates degranulation by coordinating the interactions that occur in the plasma-membrane microdomains that are required for the activation of PLCγ.

LAT, PLCγ and generation of other mediators

The generation of eicosanoids has not been examined in $Lat^{-/-}$ BMMCs; however, compared with wild-type BMMCs, there is a marked, but not complete, reduction in the increase in cytokine- and chemokine-encoding mRNA and protein concentrations that occurs in response to antigen[26]. This residual cytokine-encoding

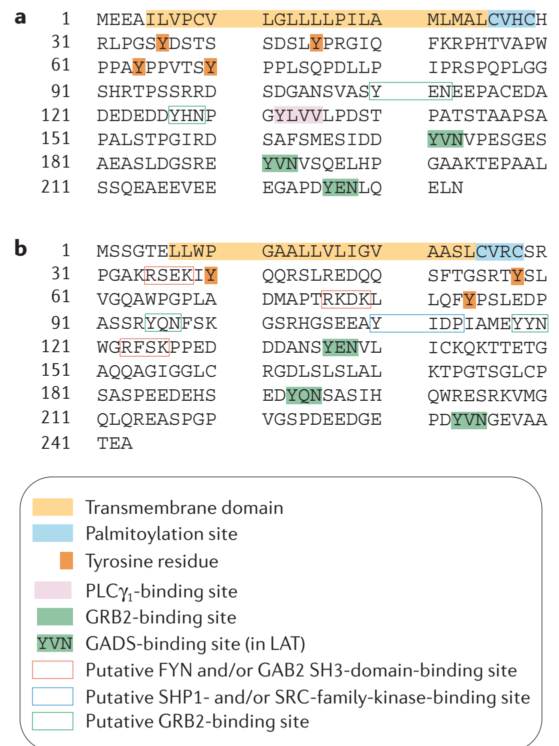

Figure 3 | **Amino-acid sequences and protein–protein interaction sites of human LAT and NTAL. a** | Binding sites for GRB2 (growth-factor-receptor-bound protein 2), GADS (GRB2-related adaptor protein) and phospholipase $C\gamma_1$ ($PLC\gamma_1$) in LAT (linker for activation of T cells) (isoform b) are shown. **b** | Putative binding sites for FYN and/or GAB2 (GRB2-associated binding protein 2), and SHP1 (SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatase 1) and/or SRC-family kinase, in NTAL (non-T-cell activation linker) are shown. Other tyrosine residues and putative GRB2-binding sites, which have not yet been shown experimentally to be phosphorylated, are also indicated.

mRNA that is observed in antigen-challenged $Lat^{-/-}$ BMMCs is again almost absent in antigen-challenged $Lat^{-/-}Ntal^{-/-}$ BMMCs[37]. The sequence of events that leads from LAT to cytokine production has not been as clearly defined as the sequence that leads to degranulation. However, the pathways that lead to cytokine-gene expression require the guanine-nucleotide-exchange factors VAV[56] (TABLE 2) and SOS[57] to shift the equilibrium of RAS from the inactive (GDP bound) state to the active (GTP bound) state[58]. After it has been activated, RAS positively regulates the RAF-dependent pathway that leads to phosphorylation and, in part, activation of the mitogen-activated protein kinases (MAPKs) extracellular-signal-regulated kinase 1 (ERK1) and ERK2 (REF. 59). The MAPKs JUN amino-terminal kinase (JNK) and p38 are similarly activated in a LAT-dependent manner in mast cells[44], but the mechanism(s) that regulates these responses is less well-defined (FIG. 2). These molecules (ERK1, ERK2, p38 and JNK), in turn, activate transcription factors — including the activator protein 1 (AP1) components

Passive systemic anaphylaxis
An experimental technique that, similar to passive cutaneous anaphylaxis, reflects mast-cell degranulation. Mice or rats are passively sensitized by intravenous injection of antigen-specific IgE, then antigen is injected into the tail vein. The concentration of histamine in the plasma, which indicates systemic mast-cell activation, is then assessed.

SRC-homology-2 domain
(SH2 domain). A protein–protein interaction domain that is commonly found in signal-transduction molecules. It specifically interacts with phosphotyrosine-containing sequences.

Immunoreceptor tyrosine-based activation motif
(ITAM). A structural motif that contains tyrosine residues and is found in the cytoplasmic tail of several signalling molecules. The motif has the form YXXL/I (where X denotes any amino acid), and the tyrosine residue is a target for phosphorylation by protein tyrosine kinases of the SRC family and, subsequently, for the binding of proteins that contain SRC-homology-2 domains.

Adaptor molecule
A signalling molecule that has one or more binding motifs and/or tyrosine residues that can be phosphorylated by SRC-family kinases, SYK (spleen tyrosine kinase) and/or ZAP70 (ζ-chain-associated protein kinase of 70 kDa). These molecules mainly function as components of a 'molecular scaffold' that organizes activated receptor signalling complexes.

Palmitoylation site
A juxtamembrane sequence (CXXC, where X denotes any amino acid) that is found in signalling proteins, including SRC-family kinases and transmembrane adaptor molecules. It allows binding of these proteins to plasma-membrane palmitic acid and thereby targets these proteins to lipid rafts.

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Small interfering RNA (siRNA). Short, double-stranded RNA molecules of 19–23 nucleotides that induce RNA interference (RNAi), a post-transcriptional process that leads to gene silencing in a sequence-specific manner.

(FOS and JUN)[60], nuclear factor of activated T cells (NFAT)[61] and nuclear factor-κB (NF-κB)[62,63] — leading to cytokine generation. Because FOS and JUN activation is regulated by PKC[64] and because the regulatory pathway for NFAT activation is calcium dependent[65], PLCγ can also regulate antigen-mediated expression of cytokine genes by mast cells. Similar to degranulation, the four terminal tyrosine residues in LAT are essential and sufficient for the ability of LAT to regulate FcεRI-dependent cytokine and chemokine production by mast cells[44]. Interestingly, the Y132 PLCγ-binding site in LAT might have an individual contribution to cytokine and chemokine production[44]. Although this site (together with the GRB2- and GADS-binding sites) seemed to contribute to the activation of ERK1, ERK2, JNK and p38, studies carried out in the Jurkat T-cell line indicate that the ability of PLCγ to regulate cytokine production is mainly a consequence of the ability of PLCγ to influence NFAT activation[31].

On the basis of this discussion, FcεRI-mediated mast-cell activation, in this simplified view, can therefore be thought of as a linear FcεRI-proximal pathway, comprising a series of sequential steps from the receptor to LYN and SYK and finally to LAT, with subsequent LAT-distal divergence to accommodate the signalling requirements for the generation and/or release of the various pro-inflammatory mediators (FIG. 2). The role of NTAL in the signalling pathway that leads to FcεRI-dependent mast-cell activation remains unclear. However, data from studies of *Lat*$^{-/-}$*Ntal*$^{-/-}$ BMMCs imply that LAT and NTAL might function in a complementary manner to regulate calcium mobilization and degranulation. Recent studies have provided evidence of a secondary pathway in activated mast cells that might complement and/or amplify what we term the principal pathway (the pathway described in this section), and it is possible that NTAL might have a role in the regulation of this pathway.

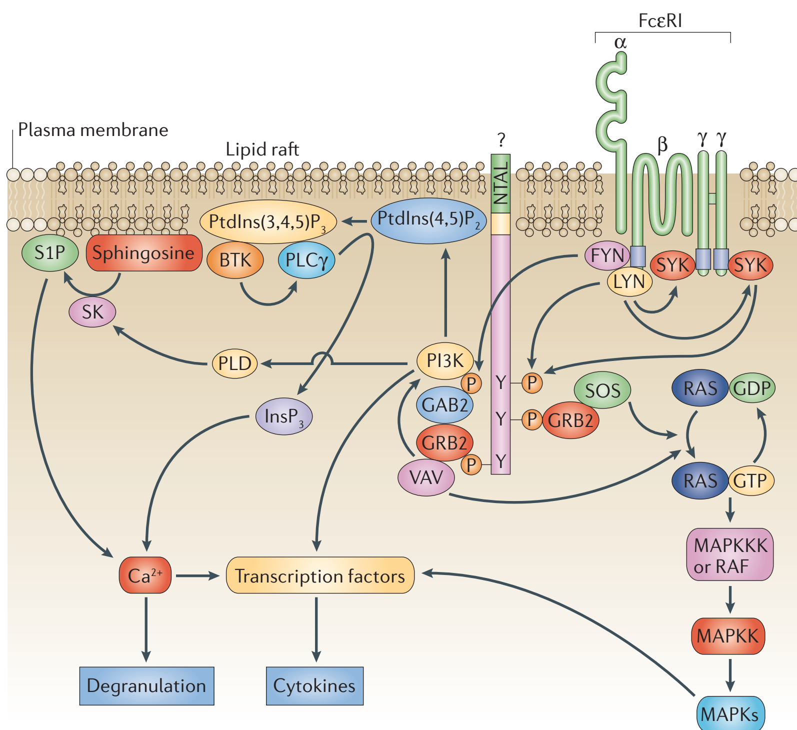

Figure 4 | **The 'complementary' (amplification) signalling cascade in activated mast cells.** For clarity, only one high-affinity receptor for IgE (FcεRI) is shown. Following FcεRI aggregation, the protein tyrosine kinase FYN becomes activated, which results in tyrosine phosphorylation of the cytosolic adaptor molecule GAB2 (growth-factor-receptor-bound protein 2 (GRB2)-associated binding protein 2). This leads to the binding of phosphatidylinositol 3-kinase (PI3K) by GAB2, resulting in an increase in calcium (Ca^{2+}) mobilization, which potentially occurs through a mechanism that involves the BTK (Bruton's tyrosine kinase)-dependent phosphorylation of phospholipase Cγ (PLCγ). Alternatively, PI3K might increase calcium mobilization by activation of sphingosine kinase (SK) through phospholipase D (PLD), resulting in the formation of sphingosine 1-phosphate (S1P). Although, it has not yet been shown, NTAL (non-T-cell activation linker) might coordinate these events by binding GRB2 following phosphorylation of NTAL in a LYN- and SYK (spleen tyrosine kinase)-dependent manner. $InsP_3$, inositol-1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate; SOS, son of sevenless homologue.

A complementary activation pathway

The FYN–GAB2–PI3K axis. The concept of an amplification signal for FcεRI-mediated mast-cell activation was originally inferred from studies showing that FcεRI-dependent mast-cell activation was only partially reduced in the absence of the FcεRI β-chain[66], whereas it was completely abolished in the absence of the γ-chains[67,68]. From these studies, and an additional study[69], it was concluded that, whereas the γ-chains are required for degranulation, the β-chain has an important role in the amplification of the signals that are initiated by the γ-chains[22,70]. The first direct evidence, however, for the existence of a complementary signalling pathway for mast-cell activation came from the laboratory of Juan Rivera. His group showed that, in addition to LYN, another SRC-family kinase, FYN, was also required for degranulation and cytokine production by activated mast cells[20]. This latter pathway did not require LAT and did not lead to the activation of PLCγ but, instead, led to the activation of phosphatidylinositol 3-kinase (PI3K) downstream of the phosphorylation of the cytosolic adaptor molecule GAB2 (GRB2-associated binding protein 2)[71] (TABLE 2; FIG. 4). Subsequently, we showed that degranulation of human mast cells was regulated by an immediate PLCγ_1-dependent but PI3K-independent pathway (the principle pathway, described earlier), as well as a latent PI3K-dependent pathway, which might maintain the initiating PLCγ_1-dependent signals[50].

PI3K phosphorylates plasma-membrane-associated phosphoinositides at the 3′ position, thereby providing a docking site for pleckstrin-homology domains of associating proteins[72]. Several signalling molecules that are important for mast-cell activation contain pleckstrin-homology domains, including PLCγ_1, PLCγ_2, VAV and the TEC-family kinase Bruton's tyrosine kinase (BTK)[29,73]. Therefore, these molecules can be recruited to the plasma membrane in a PI3K-dependent manner following FcεRI aggregation[29,73]. However, these molecules have additional binding domains, so this feature does not necessarily indicate that PI3K is essential for the function of all of these signalling molecules. In early studies carried

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out using RBL-2H3 cells, the PI3K inhibitor wortmannin was observed to inhibit both antigen-mediated calcium mobilization and degranulation effectively[49,74]. However, RBL-2H3 cells have an activating mutation in KIT that results in constitutive PI3K activation in these cells. In later studies that were carried out using cultures of primary human mast cells and wortmannin or other PI3K inhibitors (including LY294002 and IC87114)[50,75], and in studies of BMMCs from transgenic mice that have a null mutation in the p110δ subunit of PI3K[75] (TABLE 2), it was found that a proportion of the total degranulation in response to antigen was refractory to regulation by PI3K and that PI3K inhibitors were only effective at reducing the later stages of calcium mobilization[50]. Furthermore, PI3K activation was observed only after the initiating PLCγ_1 response induced by FcεRI aggregation had started to decline (~60 seconds)[50]. So, PI3K seems to be mainly responsible for the maintenance, but not the initiation, of the calcium signal that is required for optimal degranulation.

How PI3K regulates calcium mobilization in, and degranulation by, mast cells is not clear at present. For both mast cells[76] and B cells[77], it has been proposed that PI3K might regulate antigen-mediated calcium mobilization by recruiting BTK and PLCγ to the plasma membrane, where PLCγ is subsequently phosphorylated and activated by BTK (FIG. 4). However, as described earlier, at least the early PLCγ_1-dependent calcium mobilization seems to be independent of PI3K[50]. An alternative mechanism for PI3K-regulated calcium mobilization could involve the sphingosine kinase (SK)–S1P pathway. SK is activated following FcεRI aggregation at the surface of mast cells in a LYN- and FYN-dependent manner[78], and this results in the phosphorylation of lipid-raft-associated sphingosine to form S1P[79,80]. Although, as described later, S1P can be released from mast cells[81] and bind to cell-surface receptors[78], S1P has also been postulated to have an intracellular role in the regulation of mast-cell activation by inducing calcium mobilization in an $InsP_3$-independent manner[82,83]. In mast cells and other haematopoietic cells, SK activity is regulated by phospholipase D (PLD)[82]. PLD is activated in mast cells[84] in a PI3K-dependent manner[85] following FcεRI aggregation, so this might explain how PI3K can help to regulate the calcium signal in mast cells following antigen challenge.

It is unclear whether this PI3K-signalling pathway that leads to calcium mobilization and degranulation is regulated by a transmembrane adaptor molecule in a manner similar to the regulation of the PLCγ-signalling pathway by LAT. However, it has been proposed that NTAL might have this function[36,38]. In support of this hypothesis, preliminary data indicate that antigen-mediated AKT phosphorylation (a surrogate marker for PI3K activation), but not PLCγ_1 phosphorylation, is inhibited in human mast cells that are treated with *NTAL*-targeted siRNA (C.T. and A.M.G., unpublished observations). Further studies are required to delineate the potential role of NTAL or other transmembrane adaptor molecules in regulation of the FYN–GAB2–PI3K mast-cell activation pathway.

The role of PI3K in cytokine production. Because mast cells that are treated with wortmannin or other PI3K inhibitors, and BMMCs with the p110δ null mutation, have a reduced capacity to generate cytokines[75], it is clear that PI3K-dependent pathways, in addition to the LAT–PLCγ pathway, regulate FcεRI-mediated expression of cytokine genes by mast cells. Similar to the LAT-regulated pathway, the steps that lead from PI3K to the regulation of cytokine-gene transcription are less clear than those that regulate degranulation. PI3K activation, however, results in the recruitment of the serine/threonine kinase PDK1 (3-phosphoinositide-dependent protein kinase 1) to the plasma membrane, where PDK1 subsequently phosphorylates and activates another serine/threonine kinase, AKT[86]. AKT positively regulates the function of the transcription factor NF-κB by phosphorylating inhibitor of NF-κB (IκB), which is a key regulator of NF-κB[87]. In addition, because of its ability to increase calcium concentrations, PI3K might also augment the ability of PLCγ to regulate activation of the transcription factor NFAT[88]. So, PI3K might regulate cytokine production by regulating the activities of NF-κB and/or NFAT.

From the earlier discussion, we can therefore summarize that both a signalling pathway that is mediated by PLCγ_1 and regulated by LAT and a parallel signalling pathway that activates PI3K and amplifies and/or maintains the calcium signal provide crucial signals for optimal mast-cell activation. This latter pathway might be regulated by NTAL. By comparing the defects in downstream signalling in BMMCs from mice that are deficient in specific signalling molecules (TABLE 2), it has been possible to define the contribution of such molecules to these complementary signalling cascades: the principal pathway, which leads to PLCγ_1 activation, requires the FcεRI γ-chain, LAT, SLP76 and GADS; and the complementary pathway, which leads to PI3K activation, requires the FcεRI γ-chain and perhaps the β-chain, FYN, GAB2 and potentially NTAL. Interestingly, VAV seems to control both PLCγ and PI3K activation; therefore, this molecule might have a role in coordination of the responses of both the principle and the complementary pathways. The precise role of LYN in the regulation of these pathways remains unclear. Exactly how these pathways differentially regulate the activation of specific transcription factors for cytokine-gene expression also requires further study.

Physiological implications

Why should complementary signalling cascades for regulating antigen-dependent mediator release evolve in mast cells? We can postulate three answers to this question: first, these alternative pathways are 'fail-safe' mechanisms to ensure cell activation still occurs when inactivating single-nucleotide polymorphisms are present in the genes that encode crucial signalling molecules; second, these systems might provide flexibility in the signalling pathways that are required for fine-tuning mediator release; and third, as inferred from recent studies[36,89], these two complementary pathways might allow integration of signalling pathways from KIT

Protein kinase C
(PKC). A family of serine/threonine kinases that is composed of 12 isozymes in 3 distinct classes: conventional (which consists of PKCα, PKCβI, PKCβII and PKCγ), novel (which consists of PKCδ, PKCε, PKCη, PKCθ (and PKCμ) and atypical (which consists of PKCζ, PKCι and PKCλ). The activation of these enzymes is either calcium dependent (for conventional family members) or calcium independent (for novel and atypical family members). They all have a regulatory and a catalytic domain, as well as a conserved and a variable region.

Pleckstrin-homology domain
An amino-acid sequence that is present in several signalling proteins that mediate their actions through binding phosphoinositides. A subset of these domains selectively binds phosphatidylinositol-3-kinase products. Pleckstrin-homology domains also anchor proteins to membranes through the binding of membrane lipids.

TEC-family kinases
One of the three classes of protein tyrosine kinases that are required for the activation of haematopoietic cells, the other classes being the SRC- and SYK (spleen tyrosine kinase)-family kinases. The TEC-family-kinase prototypes are ITK (interleukin-2-inducible T-cell kinase) in T cells and BTK (Bruton's tyrosine kinase) in B cells. Among other functions, TEC-family kinases seem to have an important role in the activation of phospholipase Cγ after immunoreceptor ligation.

Single-nucleotide polymorphism
Typically biallelic base-pair substitutions, which are the most common forms of genetic polymorphism.

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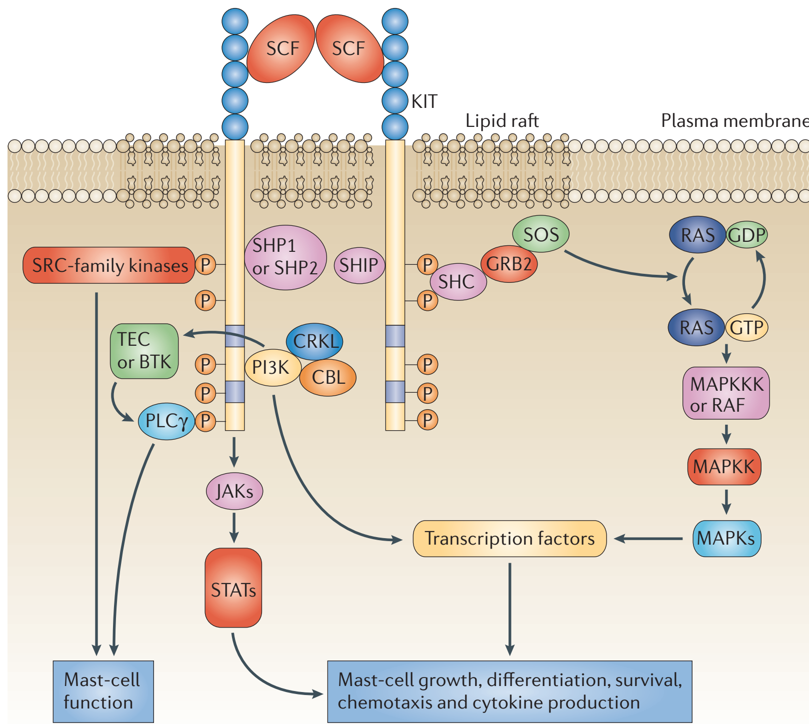

Figure 5 | **The signal-transduction molecules that are recruited and/or activated by KIT.** Stem-cell factor (SCF)-mediated dimerization of KIT induces autophosphorylation at multiple tyrosine residues in the cytoplasmic tail, resulting in the recruitment of various molecules, including the following: cytosolic adaptor molecules, such as SHC (SRC homology 2 (SH2)-domain-containing transforming protein C) and GRB2 (growth-factor-receptor-bound protein 2); SRC-family kinases; and signalling enzymes such as phospholipase Cγ (PLCγ) and phosphatidylinositol 3-kinase (PI3K). Subsequent activation of these signalling enzymes, as well as the JAK–STAT (Janus kinase–signal transducer and activator of transcription) pathway and the RAS–RAF–mitogen-activated protein kinase (MAPK) pathway leads to mast-cell growth, differentiation, survival, chemotaxis and cytokine production. As is the case for the high-affinity receptor for IgE (FcεRI), although the cascade that leads to activation of the MAPKs extracellular-signal-regulated kinase 1 (ERK1) and ERK2 is known to be regulated by RAF, the pathways by which KIT regulates the MAPK kinases (MAPKKs) and the MAPK kinases (MAPKKKs) that mediate p38 and JUN amino-terminal kinase (JNK) activation in mast cells are less well-defined. BTK, Bruton's tyrosine kinase; SHIP, SH2-domain-containing inositol-5-phosphatase; SHP, SH2-domain-containing protein tyrosine phosphatase; SOS, son of sevenless homologue; TEC, tyrosine kinase expressed in hepatocellular carcinoma.

(and by extension, from other receptors) with FcεRI-mediated signalling pathways for the regulation of mediator release.

In addition to FcεRI, receptors for other endogenous mediators are expressed by mast cells, and under the appropriate conditions, activation of these receptors might lead to an increase in basal or FcεRI-mediated mast-cell activation (TABLE 1). Although they have not been as extensively examined as the signalling cascades that are initiated by aggregated FcεRI, recent studies have now provided insight into how some of these other receptors might influence mast-cell activation.

The KIT connection. As shown using *W/W*^{v}, *W/W*^{sh} and *Sl/Sl*^{d} mice[3] and human mast cells in culture[90], SCF-dependent activation of KIT is crucial for the growth, differentiation, survival and homing of mast cells. So, FcεRI-mediated mast-cell activation *in vivo* is likely to occur on a background of SCF-mediated KIT activation. By mirroring this situation *in vitro*, studies carried out using both mouse[91] and human[36,75,92,93] mast cells have shown that SCF markedly increases degranulation in response to antigen. Although there are conflicting reports[91,92,94], our data and those of others[36,79,95,96] have concluded that SCF alone does not induce degranulation. Simultaneous addition of SCF and antigen also markedly increases the mRNA and/or protein concentrations of multiple cytokines in human[97] and mouse[96] mast cells. When added separately, however, these agents only increase basal cytokine production to a small extent[97]. For this to occur, the signalling pathways that are initiated by both receptors (that is, SCF and FcεRI) must somehow be integrated to induce the synergistic responses, and the specific signals that are required by FcεRI for inducing mast-cell-mediator release must be missing from the signalling pathway initiated by KIT.

Unlike FcεRI, KIT is a single-chain receptor that has inherent protein-tyrosine-kinase activity[40]. Nonetheless, many of the signals that are induced in mast cells by IgE-bound antigen — for example, PI3K activation, PLCγ activation, calcium mobilization and MAPK-cascade activation — are also initiated by SCF[97] (FIG. 5). At least for $PLC\gamma_1$ activation and calcium mobilization, these signals, however, are slower and of lower magnitude than those elicited by FcεRI aggregation[97]. It is also clear that these signals by themselves cannot induce degranulation[97]. The inability of SCF by itself to induce degranulation might be explained by its inability to induce a detectable increase in tyrosine phosphorylation of LAT[36] and activation of PKC[97]. By contrast, the synergistic increase in degranulation by human mast cells in response to the combination of SCF and antigen was associated with a synergistic increase in $PLC\gamma_1$ phosphorylation, calcium mobilization[97], and NTAL (but not LAT) phosphorylation[36]. A role for PI3K in this synergistic increase was indicated by studies showing that the inhibitor of the PI3K subunit p110δ (IC87114), which partially inhibits FcεRI-dependent degranulation, effectively blocked the increase in antigen-dependent degranulation by human mast cells that occurs in the presence of SCF[75]. Similarly, a role for NTAL was implied by the observation that siRNA directed to *NTAL* mRNA also abrogated the increase in antigen-induced degranulation that occurs in response to SCF[36]. Surprisingly, although SCF did not induce LAT phosphorylation in human mast cells, the ability of SCF to increase degranulation was also inhibited by siRNA directed to *LAT* mRNA, indicating that this signal, which is induced by FcεRI aggregation, is also required for synergy[36].

From these data, we can therefore conclude that SCF, in the absence of antigen, does not activate LAT-regulated pathways that are required for degranulation. This would account for the inability of SCF on its own to induce degranulation. However, SCF does activate NTAL phosphorylation and the PI3K-dependent complementary pathway. So, when the necessary LAT-mediated signals are initiated following aggregation of FcεRI, these

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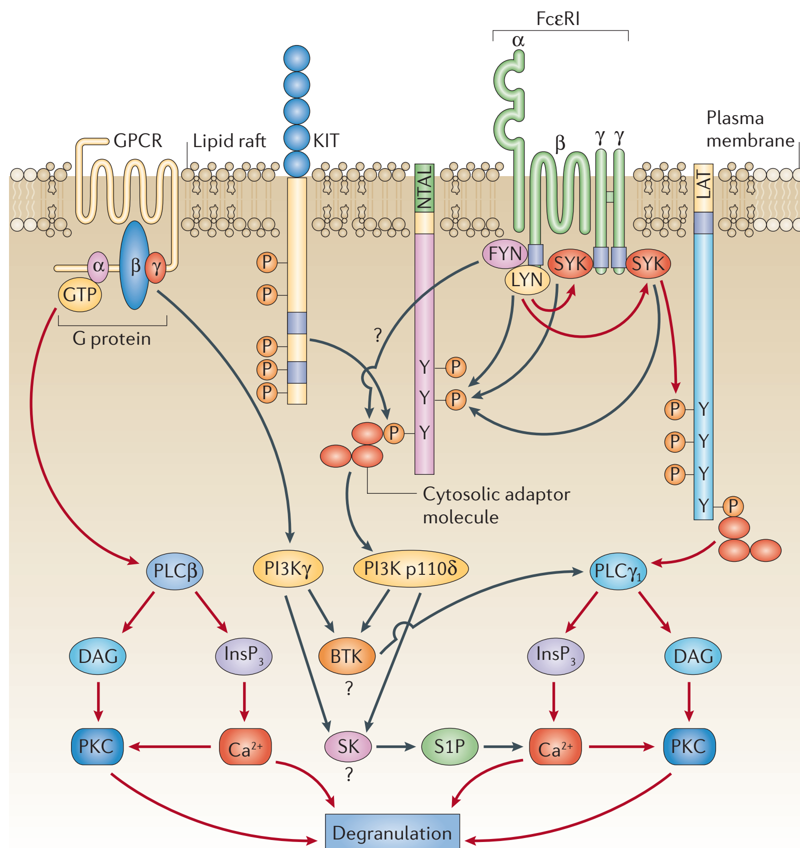

Figure 6 | **Integration of the signalling pathways induced by the G-protein-coupled receptors FcεRI and KIT.** The red arrows indicate the principle signalling pathways that are initiated by the high-affinity receptor for IgE (FcεRI), KIT and G-protein-coupled receptors (GPCRs). The black arrows indicate the complementary pathways (also termed the amplification pathways) that are used by these receptors to mediate degranulation. As indicated in FIG. 4, the role that NTAL (non-T-cell activation linker) might have in these pathways has yet to be confirmed experimentally. BTK, Bruton's tyrosine kinase; Ca^{2+}, calcium ions; DAG, diacylglycerol; $InsP_3$, inositol-1,4,5-trisphosphate; LAT, linker for activation of T cells; PI3K p110δ, phosphatidylinositol 3-kinase containing the p110δ subunit; PI3Kγ, phosphatidylinositol 3-kinase-γ; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine 1-phosphate; SK, sphingosine kinase; SYK, spleen tyrosine kinase.

W/W^{v}, *W/W*^{sh} and *Sl/Sl*^{d} mice
Mast-cell-deficient mice. These deficiencies result from a defect in cell-surface expression of KIT. *W/W*^{v} mice have a point mutation (known as viable, v) in the *Kit* gene at the dominant spotting (*W*) locus, on chromosome 5. *W/W*^{sh} mice have an inversion and a breakpoint mutation (known as sash, sh) between the genes that encode platelet-derived-growth-factor receptor and KIT. Unlike the *W/W*^{v} mice, the *W/W*^{sh} mice are fertile. For *Sl/Sl*^{d} mice, mast-cell deficiency arises from a mutation (known as Dickie, d) in the gene that encodes stem-cell factor (previously known as steel, Sl).

result in the synergistic activation of $PLCγ_1$, leading to an increase in calcium mobilization and degranulation, as observed for human mast cells[36,97] (FIG. 6).

Other receptors. The ability of mast-cell receptors to increase degranulation and/or cytokine production synergistically is certainly not restricted to FcεRI and KIT. *In vivo*, ligands for other receptors expressed by mast cells are likely to be present in the surrounding milieu, and under conditions that increase the concentration of these ligands, activation of mast cells and, in some cases, potentiation of antigen-mediated mast-cell responses can occur (TABLE 1). These receptors include adenosine receptors, the C3a receptor, S1P receptors, cytokine and chemokine receptors, and Toll-like receptors. Similar to KIT, ligation of these receptors might 'prime' activation of mast cells for subsequent antigen-mediated activation, or the ligands for these receptors might provide co-stimulatory signals for antigen-mediated responses, as has recently been suggested for the chemokines CC-chemokine ligand 3 (CCL3; also known as MIP1α) and CCL5 (also known as RANTES) when binding their cognate receptor at the surface of mast cells[98] and T cells[99], respectively. Indeed, use of CCL3-deficient mice has indicated that such interactions might be important for mast-cell-mediated responses *in vivo*[98]. How the different signalling cascades that are initiated by these receptors (TABLE 1) are integrated with those initiated by FcεRI is less clear than for KIT. However, for three of these receptors — the S1P receptor $S1P_2$, the A_3 adenosine receptor and the C3a receptor — recent studies indicate that there might be some parallels with KIT–FcεRI signal integration.

As discussed earlier, S1P is rapidly generated and secreted by mast cells following FcεRI aggregation[81,100]. In addition to its proposed intracellular role in regulating calcium mobilization, there is emerging evidence that S1P might activate mast cells in an autocrine manner after binding S1P receptors expressed by these cells. S1P receptors comprise a family of five G-protein-coupled receptors (GPCRs), two of which — $S1P_1$ and $S1P_2$ — are expressed by mast cells[81]. The expression of $S1P_2$, but not $S1P_1$, can be upregulated by mast cells following FcεRI aggregation[80]. Mast-cell-expressed $S1P_1$ induces chemotactic responses, whereas mast-cell-expressed $S1P_2$ inhibits chemotaxis but induces degranulation[81]. Accordingly, it has been proposed that antigen concentration gradients can influence mast-cell function through S1P binding to its receptors[80]. Low antigen concentrations would result in the release of S1P from the activated mast cells, thereby inducing mast-cell chemotaxis through the binding of S1P to $S1P_1$ (REF. 80). Subsequent exposure to higher concentrations of antigen would favour the induction of $S1P_2$ expression, leading to increased degranulation and chemokine production[80]. A role for $S1P_2$ in this autocrine enhancement loop for FcεRI-mediated mast-cell activation has been supported by the observation of a reduction in antigen-mediated degranulation by $S1P_2$-deficient BMMCs compared with wild-type responses[81].

When occupied by adenosine, the A_3 adenosine receptor (which is also a GPCR) similarly results in the amplification and/or maintenance of FcεRI-mediated degranulation and cytokine production[89]. In addition, when occupied by C3a, the C3a receptor (another GPCR; which can induce mast-cell degranulation and chemokine production in the absence of other stimuli[101]) synergizes with FcγRI (the high-affinity receptor for IgG; which is present at the surface of interferon-γ-treated human mast cells[102]) and potentially FcεRI to increase degranulation[89].

As described for KIT (TABLE 1), PI3K might be a key molecule in the amplification process that is associated with these GPCRs[89]. Unlike KIT and FcεRI, which are linked to class IA PI3Ks (mainly containing the p110δ subunit)[75], GPCRs — such as $S1P_2$, the A_3 adenosine receptor and the C3a receptor — are linked to class IB PI3Ks (mainly PI3Kγ)[103]. Regardless of the class of PI3K that is activated, downstream signalling events are identical. So, although it is not known whether GPCRs require

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G-protein-coupled receptor
(GPCR). One of a large group of receptors that bind a diverse set of molecules, including chemokines, complement components, bioactive amines and neurotransmitters. GPCRs are seven-transmembrane-spanning receptors and are coupled to heterotrimeric, GTP-regulated signalling proteins.

Mastocytosis
A disease that affects both adults and children and is associated with dysfunctional KIT. It is characterized by mast-cell hyperplasia in the bone marrow and peripheral tissues. The most common form is linked to an activating mutation in KIT (in which valine replaces aspartic acid at residue 816).

LAT, NTAL or a similar adaptor molecule to coordinate their influence on mast-cell activation, integration of signalling through GPCR and FcεRI ultimately occurs at the level of PI3K. As a consequence, GPCRs 'access' the complementary (amplification) pathway for mast-cell activation, albeit at a different point than is accessed by KIT (FIG. 6).

In summary, on the basis of the examples that we have described here, it can now be argued that one of the roles of the complementary (amplification) pathway for mast-cell activation is to allow signalling cascades that are initiated by other receptors, especially KIT, to be integrated with those that are initiated by FcεRI for the modulation of antigen-dependent mast-cell-mediator release. The question remains whether this paradigm is common to all receptors that regulate mast-cell activation. An answer to this question awaits further study.

Perspectives and therapeutic implications

In this Review, we have discussed the evidence for both the principle and the complementary (amplification) pathways that contribute to FcεRI-mediated mast-cell activation, and we have described how other receptors might selectively use these pathways for integrating their signalling pathways to regulate this response. Several questions, however, remain. For example, how does the concept of complementary pathways relate to disease states, and what are the implications for the development of therapeutic approaches for patients with allergy and/or other mast-cell-driven disorders? As discussed here, the antigen-mediated activation of mast cells *in vivo* is likely to be increased by other factors, such as SCF, although the relative contributions of these factors and antigen to the allergic responses that occur *in vivo* are unknown. Studies carried out *in vitro* using human mast cells, however, have shown that, even after reducing the effective antigen concentration to a point at which there is little evidence of degranulation in the absence of other stimuli, considerable degranulation still occurs if SCF is present at a physiological concentration[36]. Mast-cell-mediator release might be further increased in situations in which there are activating mutations present in receptors or signalling molecules, such as the mutation that occurs at residue 816 of KIT, as evidenced by the clinical manifestations of increased tryptase concentrations that are observed in patients with mastocytosis[104]. Similar manifestations of hypersensitive mast cells might also occur under conditions in which higher concentrations of other ligands for mast-cell receptors that increase antigen-mediated responses (such as adenosine and C3a) are present[105]. The contribution of amplification processes to mast-cell activation *in vivo* might therefore be considerable. So, targeting KIT and/or molecules of the complementary (amplification) pathway might be an attractive, or supplementary, approach for the treatment of patients with mast-cell-associated disorders.

Proof of principle for this hypothesis has come from studies showing that passive-systemic-anaphylaxis reactions and passive-cutaneous-anaphylaxis reactions are substantially reduced in GAB2-deficient mice[71] and in mice with an inactivating mutation in the p110δ subunit of PI3K[75]. Furthermore, the inhibitor of the PI3K subunit p110δ (IC87114) effectively reverses the passive-cutaneous-anaphylaxis reaction in wild-type mice[75]. These studies therefore provide impetus for designing further studies for the delineation of how the signalling pathways of other activating or synergizing receptors on mast cells are integrated for the regulation of cell activation. Furthermore, an understanding of the exact contribution of these receptors and/or pathways to mast-cell-driven disorders *in vivo* might provide a basis for the development of new strategies for the treatment of asthma and other mast-cell-linked disorders.

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Competing interests statement
The authors declare no competing financial interests.

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