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Secreted phospholipase A_2 and mast cells

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AA, arachidonic acid; PG, prostaglandin; PLA_2, phospholipase A_2; MC, mast cell; BMMC, bone marrow-derived MC; $bvPLA_2$, bee venom PLA_2; COX, cyclooxygenase; CTMC, connective-tissue MC; $cPLA_2$, cytosolic PLA_2; HDC, histidine decarboxylase; H-PGDS, hematopoietic PGD_2 synthase; $iPLA_2$, Ca^{2+}-independent PLA_2; 5-LOX, 5-lipoxygenase; L-PGDS, lipocalin-type PGD_2 synthase; LT, leukotriene; MMCs, mucosal MC; mPGES-1, microsomal PGE_2 synthase; $sPLA_2$, secreted PLA_2; SCF, stem cell factor

ABSTRACT

Phospholipase A_2s (PLA_2s) are a group of enzymes that hydrolyze the *sn*-2 position of phospholipids to release (typically unsaturated) fatty acids and lysophospholipids, which serve as precursors for a variety of bioactive lipid mediators. Among the PLA_2 superfamily, secreted PLA_2 ($sPLA_2$) enzymes comprise the largest subfamily that includes 11 isoforms with a conserved His–Asp catalytic dyad. Individual $sPLA_2$ enzymes exhibit unique tissue and cellular localizations and specific enzymatic properties, suggesting their distinct biological roles. Recent studies using transgenic and knockout mice for individual $sPLA_2$ isofoms have revealed their involvement in various pathophysiological events. Here, we overview the current state of knowledge about $sPLA_2$s, specifically their roles in mast cells (MCs) in the context of allergology. In particular, we highlight group III $sPLA_2$ (PLA2G3) as an "anaphylactic $sPLA_2$" that promotes MC maturation and thereby anaphylaxis through a previously unrecognized lipid-orchestrated circuit.
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Introduction

The mammalian genome encodes more than 30 phospholipase A_2s (PLA_2s) or related enzymes, which are classified into several structurally related families including the intracellular cytosolic PLA_2 ($cPLA_2$) and Ca^{2+}-independent PLA_2 ($iPLA_2$) families and the secreted PLA_2 ($sPLA_2$) family. The $sPLA_2$ family typically consists of low molecular mass, Ca^{2+}-requiring enzymes bearing a His–Asp catalytic dyad. Classically, $sPLA_2$s have been found abundantly in snake or insect venom. In mammals, there are 11 $sPLA_2$s (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA and XIIB), which are subdivided into a conventional group (I, II, V and X) and two atypical groups (III and XII).[1,2] Individual $sPLA_2$s have distinct substrate specificities and tissue distributions, suggesting their distinct, non-redundant roles. Indeed, recent studies using $sPLA_2$ transgenic or knockout mice have revealed that individual $sPLA_2$s exert their specific functions by producing lipid mediators, by altering the composition of membrane phospholipids, by degrading foreign phospholipids in microorganisms or dietary components, or by modifying extracellular non-cellular lipid components (lipoproteins, lung surfactant or microvesicles) in response to given microenvironmental cues. Current understanding of the *in vivo* functions of $sPLA_2$s has been summarized in recent reviews.[1–5] Here, we will make an overview of the biological roles of $sPLA_2$s and the underlying lipid pathways in allergy, focusing on mast cells (MCs) which occupy a central role in allergic responses.

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Regulation of eicosanoid generation by cPLA$_2\alpha$ in MCs

Crosslinking of the high-affinity IgE receptor FcεRI on MCs with IgE and antigen initiates signals leading to the release of allergic mediators that induce immediate hypersensitivity.[3] MCs produce prostaglandin (PG) D_2 and leukotrienes (LTs) B_4 and C_4 as lipid mediators, with preferential production of PGD_2 by connective-tissue MCs (CTMCs) and LTs by mucosal MCs (MMCs). IL-3-driven bone marrow-derived MCs (BMMCs), a relatively immature MC population, produce LTC_4 in preference to PGD_2, thus resembling MMCs. The roles of these eicosanoids in allergy are well documented by other reviews (see reviews by Kabashima and Yokomizo) in this journal issue.

Numerous studies have demonstrated that cPLA$_2\alpha$, which translocates from the cytosol to perinuclear membranes in response to an increase in cytosolic Ca^{2+} and is phosphorylated and activated by mitogen-activated protein kinases, is essential for the stimulus-coupled release of arachidonic acid (AA) from phospholipids and thereby production of eicosanoids in many cell types including MCs.[4,5] Indeed, mice lacking cPLA$_2\alpha$ (*Pla2g4a*$^{-/-}$) or those treated with a cPLA$_2\alpha$ inhibitor show attenuated asthmatic responses upon pulmonary antigen challenge,[6–8] and BMMCs from *Pla2g4a*$^{-/-}$ mice show impaired production of PGD_2, LTs and platelet-activating factor, a lysophospholipid-derived lipid mediator that also participates in allergic responses.[9,10]

Studies using IL-3-driven BMMCs as a model system have revealed that eicosanoid generation by MCs occurs in multiple phases. The immediate response, which occurs within a few minutes, involves explosive activation of cPLA$_2\alpha$ followed by prompt conversion of AA to PGH_2 and then to PGD_2 through the sequential action of cyclooxygenase (COX)-1 and hematopoietic PGD_2 synthase (H-PGDS) and to LTA_4 and then to LTC_4 through that of 5-lipoxygenase (5-LOX) in cooperation with 5-LOX-activating protein and LTC_4 synthase[11] (Fig. 1). The delayed response, which proceeds over several hours, depends entirely on sustained activation of cPLA$_2\alpha$ coupled with *de novo* induction of COX-2.[10,12,13] In a third (or priming) phase, culture of BMMCs with stem cell factor (SCF; a ligand for c-Kit) in the presence of accessory cytokines (*e.g.* IL-3, IL-4, IL-9 and IL-10) for several days not only facilitates MC proliferation, but also partially supports MC maturation toward a PGD_2-producing CTMC-like phenotype through increased expression of cPLA$_2\alpha$, COX-1 and H-PGDS.[14] On the other hand, IL-3 increases the expression of cPLA$_2\alpha$, 5-LOX and LTC_4 synthase, allowing MCs to exhibit a LTC_4-producing MMC-like phenotype.[15] Whereas LTs produced by MCs are pro-allergic,[16] PGD_2 produced by H-PGDS in MCs exhibits anti-allergic rather than pro-allergic effects in anaphylaxis and contact hypersensitivity.[17,18]

Compared to culture with SCF alone, coculture of BMMCs with fibroblasts in the presence of SCF facilitates more efficient maturation toward a CTMC-like phenotype in terms of the appearance of mature granules with greater amounts of histamine and proteases, higher cell surface expression of FcεRI, and the eicosanoid balance shift from LTs to PGD_2.[17,19,20] The latter process is characterized by increased expression of cPLA$_2\alpha$, COX-2 and H-PGDS as well as LTB_4 dehydrogenase, an enzyme that inactivates LTB_4, and decreased expression of LTA_4 hydrolase (LTB_4 synthase) and LTC_4 synthase.[21] During the BMMC-fibroblast coculture, PGE_2, which exerts an anti-allergic effect via the PGE_2 receptor EP3,[22–26] is produced by fibroblasts in a manner dependent upon cPLA$_2\alpha$ in BMMCs, where cPLA$_2\alpha$ -driven AA is transferred to adjacent fibroblasts through the transcellular route and then metabolized to PGE_2 by micosomal PGE_2 synthase (mPGES-1).[23] Mice null for mPGES-1 (*Ptges*$^{-/-}$) or EP3 (*Ptger3*$^{-/-}$) display more severe allergic responses.[22–26] Paradoxically, PGE_2-EP3 signaling induces inflammatory swelling by directly activating MCs.[27] Therefore, the anti-allergic action of PGE_2 may rely on EP3 signaling in stromal cells rather than in MCs.

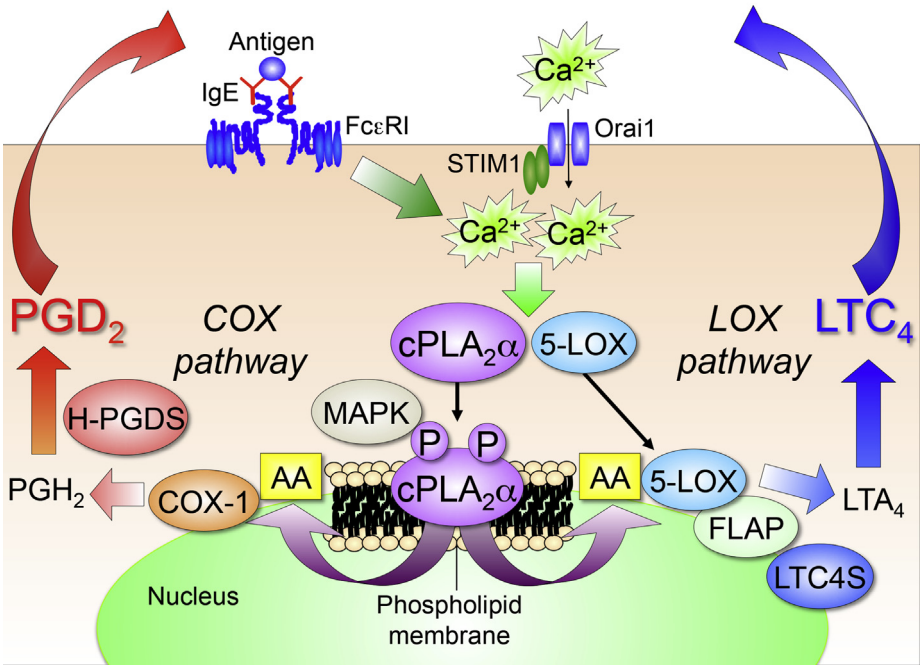

Fig. 1. cPLA$_2\alpha$-dependent eicosanoid biosynthesis in MCs. Following FcεRI crosslinking, cPLA$_2\alpha$ translocates from the cytosol to the perinuclear (preferentially Golgi) membrane in response to Stim1/Orai1-mediated Ca^{2+} influx and is phosphorylated by mitogen-activated protein kinase (MAPK) for optimal activation. The AA released from membrane phospholipids by cPLA$_2\alpha$ is then converted to PGD_2 by the sequential action of cyclooxygenase (COX)-1 (or COX-2 when the cells are primed by particular stimuli) and hematopoietic PGD_2 synthase (H-PGDS) to PGD_2 or by the sequential action of 5-lipoxygenase (5-LOX) in corporation with 5-LOX-activating protein (FLAP) and LTC_4 synthase (LTC4S) to LTC_4.

BMMCs also express iPLA$_2$β abundantly, and it has been reported that the pharmacologic inhibition of iPLA$_2$β by bromoenol lactone, a well-known iPLA$_2$ inhibitor, attenuates degranulation, eicosanoid production and cytokine release by BMMCs.[28] However, mice deficient in iPLA$_2$β (*Pla2g6*$^{-/-}$) showed a normal MC-dependent anaphylactic response *in vivo* and *Pla2g6*$^{-/-}$ BMMCs exhibit normal effector functions *in vitro*,[23] arguing against the contribution of iPLA$_2$β to MC differentiation, maturation and activation. Thus, caution should be exercised when interpreting the results obtained from studies using bromoenol lactone or any other PLA$_2$ inhibitors.

sPLA$_2$s and MCs: a historical view

It has been proposed that sPLA$_2$s, after being secreted, may act on plasma membrane phospholipids in neighboring cells (paracrine) or in their cells of origin (autocrine) to augment lipid mediator synthesis. However, this concept has yet to be substantiated because *in vivo* evidence has been largely lacking. Several reports have also suggested that sPLA$_2$s exert intracellular effects (before secretion or after internalization),[29–31] although the physiological relevance of this concept remains obscure. Despite the longer history of research on sPLA$_2$s in comparison with intracellular PLA$_2$s, the roles of sPLA$_2$s in various biological processes, including MC-dependent allergy, have remained a mystery for more than two decades.

From a historical viewpoint, sPLA$_2$ and MCs were first linked in the early 1990s, soon after the molecular identification of group IIA sPLA$_2$ (PLA2G2A or sPLA$_2$-IIA), an isoform that was originally purified from platelets or from synovial fluid of patients with rheumatoid arthritis.[32–34] As PLA2G2A (often called "inflammatory sPLA$_2$") is secreted from activated platelets, researchers have been interested in the roles of this enzyme in other hematopoietic cells including neutrophils, macrophages and MCs. Immunohistochemistry of rat serosal CTMCs has revealed the location of PLA2G2A in secretory granules,[35] although the specificity of the antibody employed in that study was unclear. We have attempted to purify PLA$_2$ enzymes expressed in BALB/c BMMCs or rat mastocytoma RBL-2H3 cells using classical column chromatography strategies and identified three distinct PLA$_2$ activities.[36] One enzyme was identified as cPLA$_2$α and another enzyme appeared to be similar to PLA2G2A in terms of several enzymatic and biochemical properties, while the entity of the third enzyme has yet to be identified. Cell biological studies have shown that degranulation rather than eicosanoid generation by rat peritoneal CTMCs is suppressed by several sPLA$_2$ inhibitors,[37,38] and exposure of these cells to very high concentrations of exogenous PLA2G2A triggers histamine release[39] or PGD$_2$ generation.[40] BMMCs rapidly internalized and degraded PLA2G2A through a heparan sulfate proteoglycan-dependent process.[41] Moreover, during the delayed response, *Pla2g2a* mRNA was induced in parallel with delayed PGD$_2$ production in BALB/c BMMCs.[42,43] However, it was subsequently found that C57BL/6 BMMCs displayed normal activation despite the intrinsic absence of PLA2G2A due to a frameshift mutation,[44] and together with the fact that the sPLA$_2$ family contains 11 isoforms,[1,2] the precise contribution of PLA2G2A to MC activation remained ambiguous.

Soon after the identification of group V sPLA$_2$ (PLA2G5 or sPLA$_2$-V) in the mid 1990s,[45] Reddy et al. reported that PLA2G5 was abundantly expressed in BMMCs and released from these cells after antigen-induced activation, acting on neighboring fibroblasts to augment COX-1-dependent PGD$_2$ biosynthesis.[46–48] This idea seemed fascinating because it was the first indication that sPLA$_2$ had a paracrine action, and it was even speculated that the role previously assigned to PLA2G2A was actually attributable to PLA2G5. Later, it was demonstrated that PLA2G2A and PLA2G5 had different intracellular locations in BMMCs, with the former existing in secretory granules and the latter in the perinuclear Golgi,[49] suggesting their distinct roles. Using BMMCs from *Pla2g5*$^{-/-}$ mice, Arm and coworkers showed that PLA2G5 amplified cytokine induction of COX-2 and attendant delayed generation of PGD$_2$ in C57BL/6, but not BALB/c, BMMCs[50] and that PLA2G5 enhanced TLR2-, but not FcεRI-dependent, eicosanoid generation in BMMCs through amplification of cPLA$_2$α activation.[51] Thus, PLA2G5 might control some MC-dependent processes related to innate immunity under certain *in vivo* conditions. However, recent studies have argued against these observations, since the expression of PLA2G5 in BMMCs proved to be rather low and MC activation both *in vivo* and *in vitro* was not affected in *Pla2g5*$^{-/-}$ mice.[17,23]

Back in the late 1990s, by which time all conventional sPLA$_2$ isoforms had been cloned,[52] we performed transfection assays of nearly a full set of conventional sPLA$_2$s using RBL-2H3 cells in order to gain insight into their potential actions in MCs. Overexpression of PLA2G2A augmented degranulation, where immunoreactive PLA2G2A was localized in secretory granules of resting cells and redistributed to sites of granular and plasma membrane fusion in activated cells, suggesting that PLA2G2A might produce fusogenic lysophospholipids *in situ*, thereby facilitating MC exocytosis.[53] Transfection of other conventional sPLA$_2$s also augmented degranulation or eicosanoid generation to various degrees.[54–56] Although these results argue for the potential roles of conventional sPLA$_2$s in MCs, their endogenous expression was undetectable or detected at only trace levels in mouse BMMCs.[17] Moreover, MC-dependent anaphylaxis was unaffected in *Pla2g2d*$^{-/-}$, *Pla2g2e*$^{-/-}$, *Pla2g2f*$^{-/-}$, *Pla2g5*$^{-/-}$ and *Pla2g10*$^{-/-}$ mice.[17] Thus, studies employing super-physiologic overexpression or addition of sPLA$_2$s do not appear to precisely reflect the physiologic, or even pathologic, situation, and the results obtained so far clearly need to be interpreted with caution. As described below, a clearer picture of the role of sPLA$_2$ in MC biology has emerged after the generation and analysis of *Pla2g3*$^{-/-}$ mice, which lack group III sPLA$_2$ (PLA2G3 or sPLA$_2$-III), an atypical sPLA$_2$ that was firstly cloned in 2000[57] and whose enzymatic and biological properties have been characterized only partially.[58,59]

Group III sPLA$_2$, a regulator of MC maturation

Bee venom PLA$_2$ (bvPLA$_2$), a major bee venom component that induces anaphylaxis, is an atypical form of sPLA$_2$.[60,61] When injected into mouse skin, bvPLA$_2$ cleaves membrane phospholipids to release lysophosphatidylcholine, which causes cell lysis, leading to activation of group 2 innate lymphoid cells by releasing the Th2-promoting cytokine IL-33.[62] The aggravated Th2-dominant IgE response to bvPLA$_2$ can be interpreted as a protective mechanism against future exposure to this noxious venom component. The sole homolog of bvPLA$_2$ in the mammalian genome is PLA2G3.[57–59,61,63,64] Given these facts, we hypothesized that PLA2G3 may function as an endogenous counterpart of bvPLA$_2$, and in fact we have found that PLA2G3 is a long-sought sPLA$_2$ that participates in the regulation of MCs.[17]

Exogenous PLA2G3, like bvPLA$_2$, elicits MC activation in mouse skin,[17] and transgenic overexpression of human PLA2G3 leads to spontaneous skin inflammation.[65] PLA2G3 is expressed more abundantly than conventional sPLA$_2$s in mouse BMMCs, where it is stored in secretory granules and released upon cell activation. Importantly, MC-associated passive and active anaphylactic responses are markedly attenuated in *Pla2g3*$^{-/-}$ mice and conversely augmented in *Pla2g3*-transgenic mice.[17] BMMCs from *Pla2g3*$^{-/-}$ mice fail to reconstitute the anaphylactic response after transfer to MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, indicating that the defect caused by

Pla2g3 deficiency is MC-autonomous. Interestingly, CTMCs in *Pla2g3*$^{-/-}$ mice are numerically normal but morphologically and functionally immature. In fact, the histamine and protease contents of granules, expression of MC maturation markers such as histidine decarboxylase (HDC, a histamine synthase) and H-PGDS, and cell surface expression of FcεRI are considerably lower in *Pla2g3*$^{-/-}$ MCs than in wild-type cells, suggesting that PLA2G3 does not merely act as a MC activator, but also facilitates MC maturation. Consistent with this, *Pla2g3*$^{-/-}$ BMMCs exhibit defective fibroblast-directed maturation, and thereby IgE-dependent and even -independent activation, releasing lower levels of histamine and PGD_2, in *ex vitro* culture. Microarray analysis has further underscored the maturation defects in *Pla2g3*$^{-/-}$ MCs (Fig. 2).

In an effort to identify the lipid mediator pathway acting downstream of PLA2G3 toward MC maturation, we used 27 knockout mouse lines deficient in biosynthetic enzymes or receptors for eicosanoids.[17] Strikingly, MC abnormalities similar to those in *Pla2g3*$^{-/-}$ mice were also seen in mice lacking lipocalin-type PGD_2 synthase (L-PGDS) or those lacking the PGD_2 receptor DP1, suggesting the functional coupling of these components in a common signaling pathway. Supporting this, pharmacological or genetic ablation of DP1 in MCs or L-PGDS in fibroblasts phenocopies that of PLA2G3 in MCs in terms of defective MC maturation and anaphylaxis. Taken together, we conclude that PLA2G3 secreted from immature MCs is coupled with fibroblastic L-PGDS to provide microenvironmental PGD_2, which in turn acts on DP1 on MCs to promote their appropriate maturation (Fig. 3).

It is well known that the SCF/c-Kit system, in cooperation with several transcription factors, adhesion molecules or accessory cytokines, is crucial for development, homing, proliferation and differentiation of MCs.[66–71] However, as SCF alone is insufficient to fully drive the terminal maturation of MCs, it has been hypothesized that some other stromal factor(s) may be additionally required. Our study has revealed that a signal driven by PGD_2 provides a missing link required for the fibroblast-driven maturation of MCs. Thus, the PLA2G3/L-PGDS/DP1 axis represents a novel lipid-orchestrated mechanism, highlighting a new aspect of PGD_2-DP1 signaling in promoting MC maturation and thereby allergy, and providing a rationale for the long-standing issue of why the secreted type of PLA_2 is needed. The paracrine PLA2G3/L-PGDS/DP1 circuit driving the proper maturation of MCs cannot be compensated by other PLA_2 subtypes, implying the specific role of this atypical $sPLA_2$.

$sPLA_2$ and human MCs: additional insights

It is important to translate the results obtained from animal studies into humans. Human skin MCs show PLA2G3 immunoreactivity, and marked expression of *PLA2G3* mRNA is detected in human lung or skin MCs.[17] Expression of *HDC* mRNA is robustly induced in human lung MCs after coculture with human lung fibroblasts, and this is suppressed by pharmacological inhibition of PLA2G3, L-PGDS or DP1. Thus, the fibroblast-directed maturation of human MCs also depends on the PLA2G3/L-PGDS/DP1 circuit.

It should be noted that MC heterogeneity in humans is more complex than that in rodents. It has been shown that PLA2G2D is weakly expressed in human cord blood-derived MCs.[72] Triggiani et al. reported that lung MCs from asthmatic patients constitutively expressed mRNAs for group IB, IIA, IID, IIE, IIF, III, V, X, XIIA, and XIIB $sPLA_2$s, and that the cell-impermeable conventional $sPLA_2$ inhibitor Me-Indoxam partially reduced the production of LTC_4 from anti-IgE-stimulated human lung MCs.[73] Considering that Me-Indoxam does not inhibit atypical $sPLA_2$s, these results suggest that some conventional $sPLA_2$s released from human lung MCs might contribute to LTC_4 production in an autocrine fashion. It remains unclear whether PLA2G3 is involved in the maturation of all MC subsets in distinct human tissues and whether other $sPLA_2$s, such as PLA2G2A, which is expressed far more abundantly than other

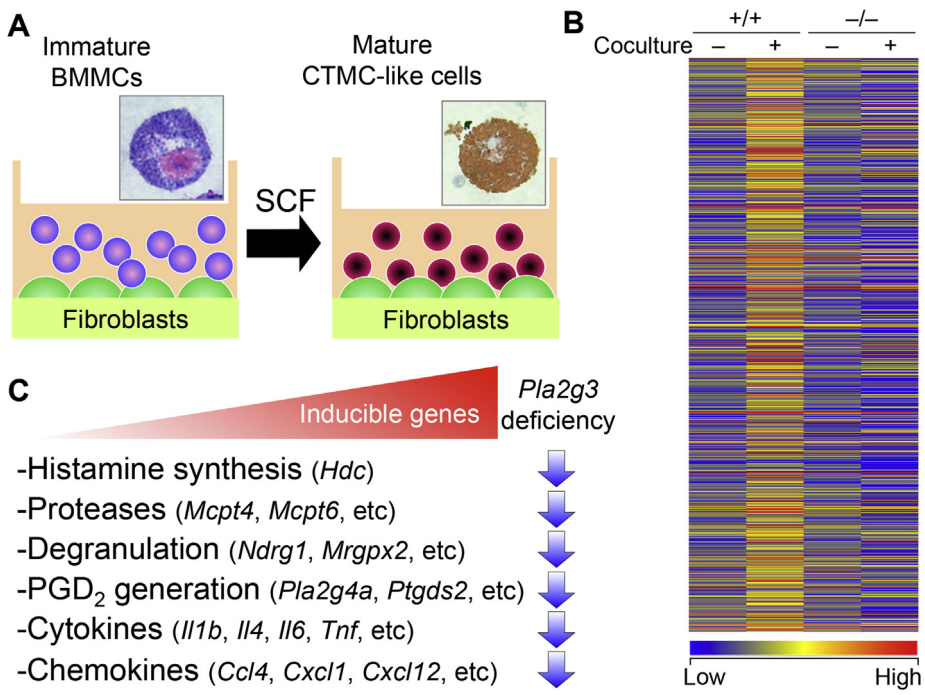

Fig. 2. Microarray analysis of Pla2g3$^{-/-}$ BMMCs in comparison with wild-type BMMCs. IL-3-driven BMMCs from Pla2g3$^{+/+}$ and Pla2g3$^{-/-}$ mice were co-cultured for 4 days with or without Swiss3T3 fibroblasts in the presence of SCF to allow maturation to CTMC-like cells (A). Of more than 40,000 genes detected so far by microarray, 3652 genes were induced in Pla2g3$^{+/+}$ CTMC-like cells compared to replicate BMMCs. Approximately 61% of these inducible genes (2223 genes) were downregulated in Pla2g3$^{-/-}$ CTMC-like cells. A heat map of the microarray analysis (B) and a list of representative genes that were affected by Pla2g3 deficiency (C) are indicated.

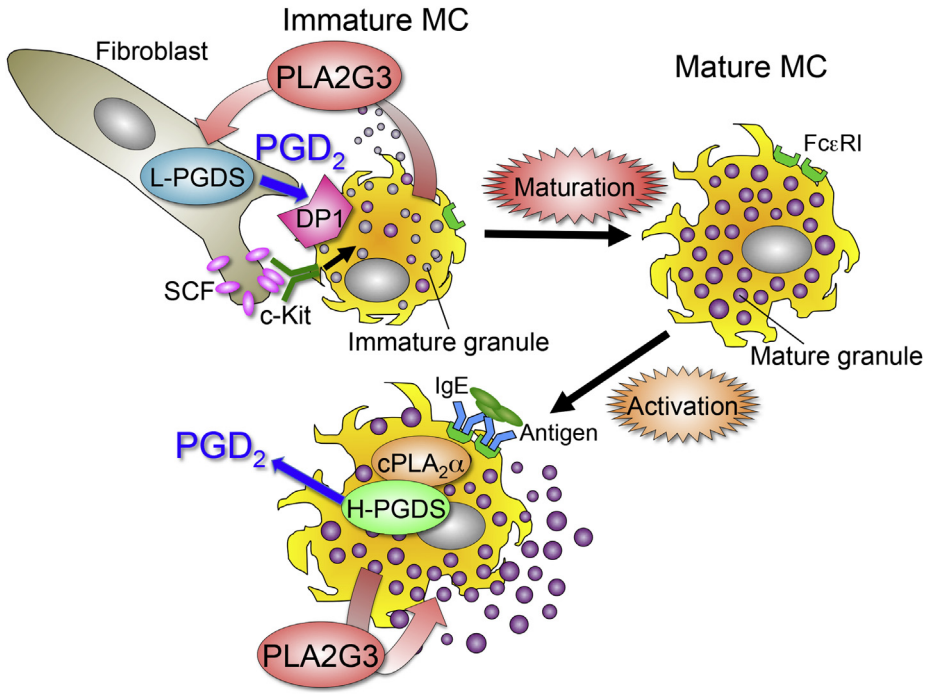

Fig. 3. Regulation of MC maturation through the paracrine PLA2G3/L-PGDS/DP1 circuit. PLA2G3 released from immature MCs acts on neighboring fibroblasts to promote L-PGDS-dependent generation of PGD_2, which in turn acts on the PGD_2 receptor DP1 on MCs to promote MC maturation. PLA2G3 released from mature MCs may also augment degranulation. As such, PLA2G3 facilitates MC-dependent anasphylaxis. Expression levels of $cPLA_2\alpha$ and H-PGDS are increased in mature MCs to supply a distinct pool of PGD_2 that negatively controls anaphylaxis.

$sPLA_2$s in human MCs from various tissues (unpublished data), could functionally substitute for PLA2G3 or have other roles.

Concluding remarks

In this article, we have provided an overview of more than 20 years of studies on $sPLA_2$s in MCs conducted by both our group and others. On the basis of the dogma prevailing in PLA_2 research, most investigators used to speculate that $sPLA_2$ might be involved in some phases of eicosanoid production by MCs, even after the central role of $cPLA_2\alpha$ in this process had been established. The discovery of PLA2G3 has greatly expanded our understanding of $sPLA_2$ as a regulator of MC maturation via a paracrine PGD_2 loop.[17] Besides the regulation of MCs and allergy, accumulating evidence suggests that $sPLA_2$ generally acts as a paracrine coordinator of various pathophysiological events by driving a unique form of lipid metabolism in response to a specific microenvironmental cue.[63,74,75]

There are, however, several questions to be answered. Firstly, is PLA2G3 the only $sPLA_2$ involved in the regulation of MCs or MC-associated allergic diseases? Since we used only limited criteria with which to evaluate the functions of MCs,[17] investigation of other parameters, such as host defense against parasites or bacteria, might reveal a yet unidentified role of PLA2G3 or other $sPLA_2$s in MCs. In fact, PLA2G2A acts as a potent "bactericidal $sPLA_2$" by degrading the cell membranes of Gram-positive bacteria,[76] and some constituents of MC granules such as proteases have a protective role against exogenous venom components.[77] Secondly, does PLA2G3 regulate more complex chronic allergic diseases such as asthma or atopic dermatitis? Although it has been reported that *Pla2g5*$^{-/-}$ or *Pla2g10*$^{-/-}$ mice exhibit reduced airway inflammation in asthma models,[78,79] the role of PLA2G3 in asthma is currently unknown. It is noteworthy that PLA2G5 is a "Th2-prone $sPLA_2$" that promotes Th2 immunity by facilitating M2 polarization of macrophages,[74,80] whereas PLA2G10 appears to be secreted from airway epithelial cells and then to act on neighboring leukocytes to augment the generation of eicosanoids.[81] Considering that MCs have been implicated in obesity and atherosclerosis,[82,83] the role of PLA2G3 in these metabolic diseases would also be worth examining. Thirdly, is PLA2G3 expressed in immune cells other than MCs? Our preliminary study has revealed the expression of PLA2G3 in non-MC immune cell populations (unpublished data), which might also affect the pathology of allergic or other immune diseases. Lastly, specific inhibitors for PLA2G3 are currently unavailable, which limits our understanding of the efficacy of targeting this enzyme for disease therapy. Given the unique structural features of PLA2G3,[84] its inhibitor would require rather strict specificity, and its application would give further insight into the unique and important roles of this atypical $sPLA_2$ in mouse disease models as well as human diseases.

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Conflict of interest
The authors have no conflict of interest to declare.

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