Mast cell maturation is driven via a group III phospholipase A2-prostaglandin D2–DP1 receptor paracrine axis

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Microenvironment-based alterations in phenotypes of mast cells influence the susceptibility to anaphylaxis, yet the mechanisms underlying proper maturation of mast cells toward an anaphylaxis-sensitive phenotype are incompletely understood. Here we report that PLA2G3, a mammalian homolog of anaphylactic bee venom phospholipase A2, regulates this process. PLA2G3 secreted from mast cells is coupled with fibroblastic lipocalin-type PGD2 synthase (L-PGDS) to provide PGD2, which facilitates mast-cell maturation via PGD2 receptor DP1. Mice lacking PLA2G3, L-PGDS or DP1, mast cell–deficient mice reconstituted with PLA2G3-null or DP1-null mast cells, or mast cells cultured with L-PGDS–ablated fibroblasts exhibited impaired maturation and anaphylaxis of mast cells. Thus, we describe a lipid-driven PLA2G3–L-PGDS–DP1 loop that drives mast cell maturation.

Anaphylaxis is a serious immediate allergic reaction that involves the activation of mast cells. Cross-linking of the high-affinity IgE receptor FcεRI on mast cells with IgE and antigen initiates signals leading to the release of allergic mediators that induce immediate hypersensitivity1. Anaphylaxis is triggered by allergens (for example, insect venom, food and medication) and damages multiple organs including the respiratory and circulatory systems, often leading to life-threatening episodes.

Environmentally induced alterations in phenotypes of mast cells could be one factor that influences the severity of anaphylaxis. Current evidence has established the essential role of stem cell factor (SCF) and its receptor c-Kit (CD117) for development of mast cells2. However, the SCF–c-Kit system alone is insufficient to drive the maturation of mast cells fully, as culture of immature mast cells with fibroblasts, but not with SCF alone, can induce differentiation into mature mast cells2. Although several cytokines, chemokines and adhesion molecules have supporting roles in tissue-specific homing, growth or differentiation of mast cells3–7, precise mechanisms underlying mast cell–fibroblast communication leading to optimal maturation of mast cells still remain elusive.

Lipid mediators, such as prostaglandins, leukotrienes and lysophospholipids, have important roles in various biological processes, including allergy8–15. A given lipid mediator (for example, PGD2) aggravates, suppresses or resolves allergic responses11–13, and this functional variability may depend on the use of distinct biosynthetic enzyme and/or receptor subtypes in different cells. Eicosanoid biosynthesis is initiated by release of arachidonic acid from phospholipids by phospholipase A2 (PLA2) enzymes16. PLA2G4A (cytosolic PLA2; cPLA2α) has an essential role in the generation of eicosanoids in various cells, and its deletion results in diminished airway hypersensitivity17.
By contrast, the role of secreted PLA₂ (sPLA₂) enzymes is still a subject of debate. Although the lower asthmatic responses in mice lacking two classical sPLA₂ enzymes (PLA2G5 and PLA2G10) have revealed their contribution to asthma, the mechanisms underlying the actions of these enzymes remain poorly understood.

A major bee venom component responsible for anaphylaxis is an atypical form of sPLA₂ called BV-PLA₂. The mammalian genome encodes group III sPLA₂ (PLA2G3), which is the sole homolog of BV-PLA₂. Here we provide evidence that PLA2G3 is a major mast cell granule-associated sPLA₂ that facilitates the maturation of mast cells by driving a previously unrecognized lipid mediator circuit. PLA2G3 released from mast cells is coupled with fibroblast lipocalin-type PGD synthase (L-PGDS) to provide PGD₂, which then acts on type-1 PGD receptor, DP1, induced on mast cells to promote their maturation.

RESULTS

PLA2G3 is expressed in mast cells and induces their activation

When injected intradermally into the mouse ear pinnas, BV-PLA₂ or human PLA2G3 alone induced a similar, dose-dependent vascular leak and augmented passive cutaneous anaphylaxis (PCA) induced by IgE and antigen in Kit⁺/− mice but not in mast cell–deficient Kit⁻/−/⁻ mice, in which the SCF receptor c-Kit has a substitution (Fig. 1a,b). The edema induced by PLA2G3 was accompanied by ultrastructural degranulation of dermal mast cells (Fig. 1c). PLA2G3 released the release of histamine (Fig. 1d), but not of lactate dehydrogenase (Supplementary Fig. 1a), from mouse peritoneal mast cells (pMCs) in a Ca²⁺-dependent manner, indicating that PLA2G3 elicits degranulation, not cell lysis.

Immunohistochemistry analysis revealed that PLA2G3 localized with toluidine blue* dermal mast cells in wild-type mice but not in Pla2g3⁻/⁻ mice (Fig. 1e). Punctate staining in resting mast cells and sparse staining in degranulated mast cells suggest that PLA2G3 is released upon degranulation (Fig. 1f). In bone marrow–derived cell populations, Pla2g3 mRNA was more highly enriched in IL-3–driven bone marrow–derived mast cells (BMMCs) and thymic stromal lymphopoietin (TSLP)-driven bone marrow–derived basophils (BM basophils) than in GM-CSF–driven bone marrow–derived dendritic cells (BMDCs) and M-CSF–driven bone marrow–derived macrophages (BMDMs), and was undetectable in Swiss 3T fibroblasts (Fig. 1g and Supplementary Fig. 1b). Of the mRNAs encoding sPLA₂ isoforms, Pla2g3 mRNA was expressed most abundantly in BMMCs, followed by Pla2g5 and Pla2g2e, whereas mRNAs encoding the other sPLA₂ isoforms were undetectable, and SCF-fibroblast–driven maturation of these cells toward connective tissue mast cells (CTMCs) did not affect the expression of these sPLA₂ enzymes (Supplementary Fig. 1c).

When we transfected rat mastocytoma RBL-2H3 cells with cDNA encoding PLA2G3 or a catalytically inactive PLA2G3 variant, III-HQ, in which the catalytic-center histidine was replaced with asparagine, release of β-hexosaminidase (β-HEX) and generation of PGD₂ by crosslinking of FcεRI by IgE and antigen (hereafter called IgE-Ag) was augmented in cells overexpressing native PLA2G3 but not catalytically inactive PLA2G3 (Supplementary Fig. 1d). Thus, PLA2G3 is the main sPLA₂ in mouse mast cells, is released by exocytosis and can augment activation of mast cells in a manner dependent upon its enzymatic activity.

Pla2g3 deletion ameliorates mast cell–associated anaphylaxis

Upon passive systemic anaphylaxis (PSA) induced by IgE-Ag, Pla2g3⁻/⁻ and WBB6F1-Kit⁺/⁺ mice, but not mast cell–deficient WBB6F1-Kit⁻/⁻ mice, had much more plasma histamine and a temporary decrease in rectal temperature after systemic antigen challenge, whereas these responses were mild in Pla2g3⁻/⁻ mice (Fig. 2a). Upon PCA induced by IgE-Ag (Fig. 2b and Supplementary Fig. 1e) or compound 48/80 (C48/80; Fig. 2c), edema was markedly lower in Pla2g3⁻/⁻ mice than Pla2g3⁺/⁺ mice. By contrast, transgenic overexpression of human...
PLA2G3 (PLA2G3<sup>+/−</sup>) augmented both IgE-Ag–dependent (Fig. 2d and Supplementary Fig. 1e) and C48/80-induced (Fig. 2e) PCA as well as IgE-Ag–induced PSA (Fig. 2f). Although the ear skin of Pla2g3<sup>+/−</sup> and Pla2g3<sup>+/−</sup> mice contained an equivalent number of toluidine blue<sup>+</sup> mast cells, we detected fewer cells showing signs of IgE-Ag–induced degranulation in Pla2g3<sup>−/−</sup> mice than in Pla2g3<sup>+/−</sup> mice (Fig. 2g). Conversely, ears of IgE-Ag–treated PLAG2<sup>3+/−</sup> mice had more degranulated mast cells than those of replicate control mice despite a similar total mast cell count (Fig. 2h). IgE-Ag–induced PCA in mice lacking other sPLA<sub>2</sub> enzymes (Pla2g2d<sup>−/−</sup>, Pla2g2c<sup>−/−</sup>, Pla2g2f<sup>−/−</sup>, Pla2g5<sup>−/−</sup> and Pla2g10<sup>−/−</sup>) was similar to that in respective wild-type littermates (Supplementary Fig. 1f).

We immunized Pla2g3<sup>+/+</sup> and Pla2g3<sup>−/−</sup> mice intraperitoneally with alum-adsorbed ovalbumin (OVA) and elicited active cutaneous anaphylaxis by intradermal injection of OVA, which cross-links endogenous IgE-bound FcεRI on mast cells. Under conditions in which serum anti-OVA IgE levels were similar in both genotypes, Pla2g3<sup>−/−</sup> mice exhibited lower local anaphylaxis than did Pla2g3<sup>+/+</sup> mice, as indicated by notable reductions in ear swelling and mast cell degranulation (Fig. 2i–k). Thus, PLA2G3 is the sole sPLA<sub>2</sub> isoform associated with mast cell–dependent anaphylaxis.

**Pla2g3 deletion impairs maturation of tissue mast cells**

Transmission electron microscopy analysis revealed that resting mast cells in Pla2g3<sup>+/−</sup> mice were oval with regular short processes and had many secretory granules filled with electron-lucent and dense contents, whereas those in Pla2g3<sup>−/−</sup> mice had unusual granules that were small and irregular in size, suggesting the...
immaturity of mast cells (Fig. 3a and Supplementary Fig. 2a). After challenge with antigen, Pla2g3+/+ skin mast cells exhibited features typical of degranulation, whereas Pla2g3−/− mast cells were almost insensitive. In agreement, the amount of histamine (Fig. 3b) and the expression of Hdc (which encodes histidine decarboxylase, a histamine-biosynthetic enzyme; Fig. 3c) were lower in the ears of Pla2g3−/− mice than in those of Pla2g3+/+ mice. Enzymatic activity (Fig. 3d) and expression (Fig. 3e) of mast cell proteases, including chymase (encoded by Mcpt4), tryptase (encoded by Mcpt6) and carboxypeptidase (encoded by Cpa3), were also notably lower in the ears of Pla2g3−/− mice relative to Pla2g3+/+ mice. However, expression of Kit, Mitf (which encodes a transcription factor essential for mast cell differentiation) and Srgn (which encodes serglycin, a proteoglycan core protein) was unchanged in ears of Pla2g3−/− mice (Fig. 3e), indicating that not all mast cell markers were affected by PLA2G3 deficiency. We confirmed the lower expression of HDC and the unaltered expression of c-Kit in the skin of Pla2g3−/− mice by immunoblotting (Fig. 3f).

Pla2g3−/− pMCs also had smaller and more irregular granules (Fig. 3g and Supplementary Fig. 2b), contained less histamine (Fig. 3h) and exhibited less IgE–Ag–induced histamine release (both amount and percentage; Fig. 3i) than Pla2g3+/+ pMCs. Although the proportion of Kit+FcεRI+ skin mast cells or pMCs was similar in both genotypes, surface expression of FcεRIα was lower in Pla2g3−/− mice than in Pla2g3+/+ mice (Supplementary Fig. 2c,d). A23187-induced histamine release by Pla2g3−/− pMCs was lower in terms of amount, but not percentage, compared to that by Pla2g3+/+ pMCs (Supplementary Fig. 2e), suggesting that the attenuated IgE–Ag–induced degranulation and anaphylaxis in Pla2g3−/− mice was mainly due to the lower histamine content and surface FcεRI expression. Furthermore, intestinal expression of Mcpt1 and Mcpt2 (which encode mucosal mast-cell proteases) was markedly
lower in *Pla2g3−/−* mice than in *Pla2g3+/+* mice (Supplementary Fig. 2f). Thus, the lower anaphylaxis in *Pla2g3−/−* mice may result from abnormalities in the maturation and degranulation of mast cells in multiple anatomical sites. Other immune-cell populations in the skin and spleen were unaffected by *Pla2g3* deficiency (Supplementary Fig. 2g,h).

To assess whether the aberrant features of mast cells in *Pla2g3−/−* mice relied on the absence of *PLA2G3* in the mast cells themselves or in mast cell microenvironment, we transferred *Pla2g3−/−* or *Pla2g3+/+* BMMCs intradermally into mast cell–deficient *KitW−sh/W−sh* mice. After 6 weeks, the distribution of mast cells in the ear dermis was comparable between mice reconstituted with *Pla2g3+/+* BMMCs and those reconstituted with *Pla2g3−/−* BMMCs (Supplementary Fig. 3a).

Expression of mast-cell marker genes *Hdc*, *Mcpt4*, *Mcpt6* and *Cpa3* (Fig. 3i) and IgE-Ag–mediated PCA (Fig. 3k and Supplementary Fig. 3b) was much greater in the ears of mice that received *Pla2g3+/+* BMMCs over control *KitW−sh/W−sh* mice, whereas these changes were scarcely seen in mice that received *Pla2g3−/−* BMMCs. We observed similar results when we transferred *Pla2g3+/+* or *Pla2g3−/−* BMMCs intravenously into *KitW−sh/W−sh* mice. After 12 weeks of reconstitution, IgE-Ag–mediated PCA was restored in mice reconstituted with *Pla2g3+/+* BMMCs but remained poor in mice reconstituted with *Pla2g3−/−* BMMCs, although we observed similar numbers of reconstituted mast cells in the ear dermis (Supplementary Fig. 3c,d). In these experiments, low levels of mast-cell engraftment in the skin of *KitW−sh/W−sh* mice relative to baseline amounts in the skin of wild-type mice restored PCA efficiently. *KitW−sh/W−sh* mice transfected with *PLA2G3* had a greater PCA response compared to those transfused with control BMMCs (Supplementary Fig. 3e). Altogether, the defective maturation and activation of mast cells in *Pla2g3−/−* mice are cell autonomous, even though the migration of mast cell progenitors into extravascular tissues is not profoundly impaired by *Pla2g3* deficiency.

**Impaired maturation of *Pla2g3−/−* mast cells in culture**

*Pla2g3−/−* BMMCs grew normally in medium supplemented with IL-3 (Supplementary Fig. 4a) and, unlike tissue-resident mast cells, they had normal surface expression of FcεR1α (Supplementary Fig. 4b). Stimulation with IgE-Ag induced a robust release of sPLA2 activity from wild-type BMMCs, whereas this release was ablated in *Pla2g3−/−* BMMCs and augmented in *PLA2G3* BMMCs (Supplementary Fig. 4c,d), confirming that *PLA2G3* is released upon degranulation. IgE-Ag–stimulated *Pla2g3−/−* BMMCs released less histamine, PGD2 and LTC4 than *Pla2g3+/+* BMMCs, whereas these responses were greater in *PLA2G3* BMMCs than in control BMMCs (Supplementary Fig. 4e–j). IgE-Ag–induced influx of Ca2+ induction of cytokines (encoded by *Il4*, *Il6* and *Tnf*) and phosphorylation of phospholipase C (PLCγ2) and Akt were similar between the genotypes (Supplementary Fig. 4k–m), suggesting that FcεRI-dependent signaling was not profoundly perturbed by *PLA2G3* deficiency. Generation of eicosanoids by mast cells depends on fibroblasts. Histamine content and decrease in arachidonic acid–containing phosphatidylcholine were partially impaired in *Pla2g3−/−* BMMCs compared to *Pla2g3+/+* BMMCs, despite the equivalent expression of total ERK and cPLAα proteins in both cells (Supplementary Fig. 4n–o). Thus, *PLA2G3* deficiency attenuates activation of ERK and cPLAα in BMMCs.

We took advantage of an *in vitro* system in which immature BMMCs undergo maturation toward mature CTMC-like cells in coculture with Swiss 3T3 fibroblasts27. *PLA2G3* deficiency did not affect the proliferation of BMMCs in coculture (Supplementary Fig. 5a). During coculture, sPLA2 activity was secreted from wild-type BMMCs in response to SCF, whereas sPLA2 secretion was absent in *Pla2g3−/−* BMMCs and augmented in *PLA2G3* BMMCs (Fig. 4a,b). Although the ultrastructure of *Pla2g3−/−* BMMCs appeared normal, *Pla2g3−/−* CTMC-like cells contained unusual granules with less electron-dense contents than did *Pla2g3+/+* CTMC-like cells (Fig. 4c and Supplementary Fig. 5b). After coculture, the expression of *Hdc* (Fig. 4d) and its product histamine (Fig. 4e) were markedly greater in *Pla2g3+/+* CTMC-like cells, whereas these changes were barely seen in *Pla2g3−/−* cells. Even before coculture, *Hdc* expression and histamine content were slightly lower in *Pla2g3−/−* BMMCs than in *Pla2g3+/+* BMMCs, indicating that some early developmental process had already been perturbed by *PLA2G3* deficiency. IgE-Ag–induced histamine release was greater in *Pla2g3+/+* cells after coculture than before coculture, whereas this coculture-driven increase in histamine release was impaired in *Pla2g3−/−* cells (Fig. 4f). Conversely, coculture-induced *Hdc* expression was greater in *PLA2G3* BMMCs than in control cells (Supplementary Fig. 5c). Supplementation with *PLA2G3* in coculture significantly restored the histamine level in *Pla2g3−/−* CTMC-like cells and also elevated it in *Pla2g3+/+* cells (Fig. 4g). *Pla2g3−/−* BMMCs without coculture did not respond to *PLA2G3* (Fig. 4g), suggesting that the action of *PLA2G3* on histamine synthesis in mast cells depends on fibroblasts. Histamine content in *Pla2g3+/+* BMMCs without coculture was substantially lower in the presence of *PLA2G3* than its absence (Fig. 4g), which might reflect that the enzyme elicits the release of prestored histamine by degranulation (Fig. 1b).

The maturation of wild-type BMMCs to CTMC-like cells increased FcεRI-dependent PGD2 synthesis (Fig. 4h), with a concomitant increase in *Ptdgs2* (hematopoietic PGD2 synthase; H-PGD2) (Fig. 4i). However, these changes in the PGD2 pathway occurred only weakly in *Pla2g3−/−* cells. Surface expression of FcεRIα was significantly elevated in *Pla2g3+/+* cells but not in *Pla2g3−/−* cells after coculture (Supplementary Fig. 5d), consistent with the lower surface FcεRIα expression on tissue-resident mast cells in *Pla2g3−/−* mice. The coculture-driven induction of *Mcpt4* and *Mcpt6* (which encode mast cell proteases) and *Ndir1* (which encodes a mast cell granule–associated protein)27 was also impaired in *Pla2g3−/−* cells, whereas the constitutive expression of *Srgn* and *Kit* was unaffected (Supplementary Fig. 5e). We verified the attenuated induction of *Hdc* and H-PGD2 and the unaltered expression of c-Kit in *Pla2g3−/−* CTMC-like cells at the protein level (Fig. 4i). Although *Pla2g3+/+* CTMC-like cells acquired sensitivity to C48/80 after coculture27, C48/80-induced degranulation (Fig. 4k) and induction of the putative C48/80 receptors encoded by *Mrgrp1x* and *Mrgrp2* (ref. 28; Supplementary Fig. 5e) after coculture were lower in *Pla2g3−/−* cells. The coculture-dependent decrease in *Igα* (which encodes integrin *α5*) and increase in *Icam1* (which encodes integrin *β1*), which participate in tissue homing of mast-cell progenitors29, were unaffected by *PLA2G3* deficiency (Supplementary Fig. 5e), consistent with the unaltered number of mast cells in *Pla2g3−/−* tissues. Microarray gene profiling using *Pla2g3+/+* and *Pla2g3−/−* BMMCs before and after coculture revealed that, of the ~41,000 genes examined, *Pla2g3+/+* cells expressed 3,632 coculture-inducible genes, of which 1,409 genes were barely or only partially induced in *Pla2g3−/−* cells. Genes affected by *Pla2g3* ablation included, for example, genes associated with secretory granules, genes related to biosynthesis or receptors for lipid mediators.
and genes for cytokines, chemokines and their receptors (Fig. 4I and Supplementary Table 1), underscoring the immaturity of Pla2g3−/− cells, particularly after coculture.

By comparison, Pla2g4a−/− mice exhibited normal IgE-Ag–induced PCA, with normal counts of dermal mast cells and normal amounts of histamine (Supplementary Fig. 5f–h). IgE-Ag–induced histamine release, cellular histamine content and Hdc expression were unaffected by ablation of cPLA2α (Supplementary Fig. 5i–k). Neither PGD2 nor LTC4 was produced by Pla2g4a−/− BMMCs (Supplementary Fig. 5l,m), confirming the obligatory role of cPLA2α in eicosanoid synthesis in mast cells28. Thus, the absence of mast cell–derived eicosanoids by cPLA2α deficiency did not affect maturation, degranulation and anaphylaxis of mast cells, suggesting that the effect of PLA2G3 deficiency on mast cells could not be simply explained by defective synthesis of eicosanoids by mast cells.

**PGD2-DP1 signals mast-cell maturation downstream of PLA2G3**
To identify the specific lipid-mediator pathway that lies downstream of PLA2G3, we induced IgE-Ag–dependent PCA on mouse lines deficient in various eicosanoid receptors or biosynthetic enzymes. Of the eicosanoid receptor–deficient mouse lines tested, PCA was lower only in mice lacking the PGD receptor DP1 (Ptgdr−/−)9. Vascular leakage was lower in ear mast cells exhibited poor degranulation despite an unaltered total count in Ptgdr−/− mice compared to wild-type mice (Fig. 5a,b). Dermal mast cells in Ptgdr−/− mice had fewer mature secretory granules, contained less histamine and were less sensitive to IgE-Ag–induced degranulation than those in Ptgdr+/+ mice (Fig. 5c,d). Whereas the PCA was efficiently restored in KitW−sh/W−sh mice reconstituted with Ptgdr+/+ BMMCs, it was restored only partially in those mice reconstituted with Ptgdr−/− BMMCs (Fig. 5e). The PCA was unaltered or only slightly augmented in mice lacking other
ecosanoid receptors or biosynthetic enzymes (Ptgdr2−/−, Ptger1−/−, Ptger2−/−, Ptger3−/−, Ptger4−/−, Ptger5−/−, Tbxax2r−/−, Lib4r−/−, Lib5−/−, Ptges−/−, Ptges2−/− and Aloxi15−/−; Supplementary Fig. 6a). Although Licks−/− mice exhibited a lower PCA response as reported10, their ear histamine content was unaffected (data not shown). Thus, abnormalities in mast cells observed in mice lacking PLA2G3 were phenocopied only in mice lacking DP1.

Next we examined the expression and function of DP1 in a mast cell–fibroblast coculture system11. Although we barely detected Ptgdr mRNA in BMMCs and Swiss 3T3 fibroblasts, Ptgdr mRNA was robustly induced in Pla2g3−/−, but not in Pla2g3−/−, CTMC-like cells after coculture (Fig. 5). Consistently, Ptgdr expression in the ear was lower in Pla2g3−/− mice than in Pla2g3+/+ mice (Fig. 5g). In agreement with the lower histamine amount in Ptgdr−/− dermal mast cells (Fig. 5d), the coculture-driven Hdc induction was severely impaired in Ptgdr−/− CTMC-like cells (Fig. 5h). In addition, the DP1 antagonist BW 245C significantly enhanced Hdc induction in wild-type CTMC-like cells (Fig. 5i). Conversely, the DP1 agonist BW 245C significantly enhanced Hdc induction in Ptgdr+/+ WT mice (Fig. 5j). However, the coculture-driven Hdc expression was barely restored by BW 245C in Pla2g3−/− mice (Fig. 5j), likely because DP1 induction was blunted by PLA2G3 deficiency (Fig. 5f). To circumvent this problem, we used the cAMP-elevating agent forskolin because DP1 is coupled with Gs-cAMP signaling5. The addition of forskolin to the coculture bypassed the requirement for DP1 in the induction of Hdc in Pla2g3−/− CTMC-like cells (Fig. 5k). By comparison, the expression of Ptgdr2, which encodes another PGD2 receptor known as CRTH2, was high in BMMCs and lowered in accordance with their maturation into CTMC-like cells, without being affected by the Pla2g3 genotypes (Supplementary Fig. 6b). Moreover, Hdc induction in CTMC-like cells was unaffected by CRTH2 deficiency in coculture, and Ptgdr2 expression was unaffected by PLA2G3 deficiency in vivo (Supplementary Fig. 6c,d). The coculture-driven production of other eicosanoids such as 15-HETE and PGA2 was unaffected by PLA2G3 deficiency (Supplementary Fig. 6e). Thus, DP1–AMP signaling is specifically required for the PLA2G3-dependent maturation of mast cells.

L-PGDS supplies a PGD2 pool for mast-cell maturation

We hypothesized that the absence of PGD2, biosynthetic enzyme(s), acting downstream of PLA2G3 and upstream of DP1, would also influence maturation of mast cells. Of the two PGD2 synthase–encoding genes, Ptgsd2 (which encodes H-PGDS) was expressed in BMMCs but not in Swiss 3T3 fibroblasts, whereas Ptgsd (which encodes lipocortin-type PGD2; L-PGDS) expression was higher in fibroblasts than in BMMCs (Fig. 6a) and was below the detection limit in pMCs (data not shown). L-PGDS immunoreactivity was associated with fibroblasts surrounding toluidine blue+ mast cells in mouse skin (Supplementary Fig. 7a). PCA was exacerbated in Ptgsd2−/− mice10, which lack H-PGDS (Fig. 6b), whereas it was suppressed in Ptgsd−/− mice13, which lack L-PGDS (Fig. 6c), in comparison with respective control mice. Ptgsd−/− mice had fewer degranulated ear mast cells than did Ptgsd+/− mice after antigen challenge, although the total mast cell count was unaffected (Fig. 6d). Dermal mast cells in Ptgsd−/− mice were ultrastructurally immotile (that is, cytoplasmic granules were small and heterogeneous), comparatively resistant to antigen-induced degranulation, and contained less histamine than those in Ptgsd+/− mice (Fig. 6e,f). Thus, the notable similarity among Pla2g3−/−, Ptgsd−/− and Ptgdr−/− mice suggests that PLA2G3, L-PGDS and DP1 may lie in the same genetic pathway.
regulatory pathway driving maturation of mast cells. The transfer of Ptgsd1+/+ or Ptgsd2−/− BMMCs into KitW−/−KitW−/− mice fully restored the PCA response (Supplementary Fig. 7b), and a similar induction of Hdc occurred when Ptgsd1+/+ or Ptgsd2−/− BMMCs were cultured with fibroblasts (Supplementary Fig. 7c), indicating that L-PGDS in fibroblasts, not in mast cells, may be important for the regulation of mast cells.

Coculture with L-PGDS–silenced Swiss 3T3 fibroblasts by two distinct Ptgsd–specific small interfering (si)RNAs resulted in less PLA2G3 supply arachidonic acid to L-PGDS in fibroblasts, it also contributed to induced expression of L-PGDS for efficient biosynthesis of a pool of PGD2 that promotes the maturation of mast cells. Thus, addition of PLA2G3 or BV-PLA2 alone did not increase PGD2 synthesis (Supplementary Fig. 7i). Conversely, induction of Ptgsd in Swiss 3T3 fibroblasts was enhanced in coculture with PLA2G3tg/+ BMMCs relative to wild-type BMMCs (Supplementary Fig. 7f–h). Additionally, we observed robust upregulation of Ptgsd2 in BMMCs and Ptgsd in Swiss 3T3 fibroblasts or in primary mouse skin fibroblasts (and to a much lesser extent in BMMCs) in wild-type BMMC cocultures, whereas these responses occurred only partially in Pla2g3−/− BMMC cocultures (Supplementary Fig. 7i–l). Thus, not only did mast cell–derived PLA2G3 supply arachidonic acid to L-PGDS in fibroblasts, it also contributed to induced expression of L-PGDS for efficient biosynthesis of PGD2 that promotes the maturation of mast cells.

However, addition of PLA2G3 or BV-PLA2 alone did not increase Ptgsd expression in fibroblasts (Supplementary Fig. 7j), suggesting that some additional mast cell–derived factor(s) may be required for the induction of L-PGDS in fibroblasts. In agreement with the in vitro studies, amounts of PGD2 (Fig. 6k) and expression of two PGDSs (Fig. 6l) were significantly lower in the ear of Pla2g3−/− mice than that of Pla2g3+/+ mice, confirming the coupling of PLA2G3 with PGD2 synthesis in vivo. Thus, PLA2G3 secreted from mast cells is staggered in nature and does not necessarily follow a single theme throughout.
Figure 7  The PLA2G3–L-PGDS–DP1 axis facilitates maturation of human mast cells. (a) Immunohistochemistry analysis of human skin sections (atopic dermatitis) with anti-PLA2G3 (α-PLA2G3) or a preimmune antibody, followed by counterstaining with toluidine blue (scale bars, 50 μm). Blue and red arrows indicate resting and degranulated mast cells, respectively. Boxed areas are magnified below (scale bars, 5 μm). (b) Expression of PLA2G3 relative to HRPT1 in primary mast cells and fibroblasts obtained from human skin and lung (n = 3). (c) Expression of HDC relative to KIT in human lung mast cells before or on day 4 of coculture with human lung fibroblasts in the presence or absence of 5 μg/ml anti-PLA2G3, 10 μM AT-56 or 1 μM BW A868C (n = 4). Data are from one experiment (b,c; mean ± s.e.m., *P < 0.05; **P < 0.01). Images in a are representative of two experiments.

PLA2G3 PGD2 axis induces maturation of human mast cells

In human skin, toluidine blue+ dermal mast cells showed PLA2G3 immunoreactivity, although some toluidine blue+ cells also appeared PLA2G3− (Fig. 7a). We detected PLA2G3 mRNA expression in mast cells in preference to fibroblasts obtained from human lung and skin (Fig. 7b). HDC mRNA expression was robustly induced in human lung mast cells after coculture with human lung fibroblasts, and this induction was suppressed either by anti-PLA2G3, by L-PGDS inhibitor (AT-56) or by DP1 antagonist (BW A868C) to a similar extent (Fig. 7c). Thus, the fibroblast-dependent HDC induction in human mast cells also depends on the PLA2G3–L-PGDS–DP1 pathway.

DISCUSSION

Here we showed that PLA2G3, a major sPLA2 in mast cells, contributed to anaphylaxis by inducing maturation of mast cells in concert with adjacent fibroblasts through a unique pathway involving a cell-to-cell loop of the biosynthetic and receptor pathway for PGD2. Promotion of mast cell maturation by PGD2–DP1 signaling provides a mechanistic explanation for the protective effect of systemic DP1 ablation on asthma. The paracrine PGD2 circuit driven by PLA2G3, an ‘anaphylactic spPLA2’, is a previously unidentified lipid-orchestrated pathway linked to allergy and uncovers a missing microenvironmental cue underlying the proper maturation of mast cells. The SCF–c-Kit system, in cooperation with transcription factors, integrins or accessory cytokines, is essential for the development, homing, proliferation and differentiation of mast cells3–7. However, SCF alone is insufficient to drive the full maturation of mast cells, leading to the hypothesis that some other stromal factor(s) may be additionally required. These signals may include, for instance, interleukin 33, nerve growth factor, the morphogen TGF-β, hyaluronic acid and the adhesion molecule SgIGSF (spermatogenic immunoglobulin superfamily)3,4,7, although their in vivo relevances have not yet been fully understood. As in mice lacking PLA2G3, mast cells in mice lacking histamine (Hdc−/−) or heparin (Ndst2−/− or Srgn−/−) are immature and have low histamine content32–34, suggesting that the lower amount of histamine may underlie, at least in part, the defective maturation of mast cells. We showed here that a signal driven by PGD2, a bioactive lipid, is a missing link required for the fibroblast-driven maturation of mast cells. The PLA2G3–L-PGDS–DP1 circuit revealed the paracrine action of sPLA2 in the biosynthetic mobilization of PGD2 by proximal cells, the functional segregation of the two PGDS enzymes in distinct cell populations and a new aspect of PGD2–DP1 signaling in promoting maturation of mast cells and thereby allergy. Moreover, our results revealed a previously unidentified aspect of the stromal cytokine SCF, which triggers this unique lipid-driven pathway by inducing PLA2G3 secretion from mast cells.

L-PGDS, a fibroblast-cell enzyme, acts downstream of PLA2G3 to supply PGD2 to DP1 in mast cells to drive their terminal maturation. Contrary to our prediction, PGD2 driven by H-PGDS, a mast cell enzyme, had an anti-allergy role, a view that is consistent with the exacerbated allergen-induced contact dermatitis in Ptgds−/− mice11. Although it is unclear how the L-PGDS–driven extrinsic, but not the H-PGDS–driven intrinsic, pool of PGD2 is preferentially used by DP1 on mast cells, we speculate that the prolonged supply of PGD2 by L-PGDS, rather than its transient supply by H-PGDS, may be suitable for a long-lasting cell differentiation process. Alternatively, the PGD2 captured by L-PGDS, a lipid carrier protein (lipocalin), may be stabilized or better presented to mast cell DP1. This idea is reminiscent of a finding that lysophosphatidic acid (LPA), another lipid mediator, is presented to its cognate receptor as a complex with autotaxin, an LPA-producing enzyme35. The spatiotemporal discrimination of distinct PGD2 pools is also supported by the fact that although PGD2 promotes Th2-based asthma8, it contributes to resolution of inflammation through limiting neutrophil infiltration, dendritic cell activation or other mechanisms11–13.

The paracrine PLA2G3–L-PGDS–DP1 circuit could not be compensated by other PLA2 enzymes, implying a specific role of this atypical sPLA2. We observed no defects in maturation of mast cells or anaphylaxis even in mice lacking cPLA2α, although mild developmental changes in Pla2g4a−/− BMMCs have been reported, probably because of different culture conditions29. Presumably, ablation of only the specific and local lipid mediator pathway by PLA2G3 deficiency, in contrast to ablation of bulk eicosanoids in both mast cells and microenvironments by cPLA2α deficiency36, may have a different impact on mast cell phenotypes. The phenotypes observed in Pla2g3−/− mice tend to be more severe than those observed in Ptgdr−/− or Ptgds−/− mice, suggesting that PLA2G3 might be also coupled with other lipid signal(s) that could act in concert with the L-PGDS–DP1 axis to promote full maturation of mast cells. Such lipid candidates include LPA and lysophosphatidylserine, which can affect mast cell development and activation36,37. Not only can lysophospholipids transmit signals through their specific receptors, but they can also facilitate the opening of Ca2+ channels, which might explain the degranulation-promoting effect of PLA2G3 on mast cells.

Although it has been proposed that sPLA2 enzymes, after being secreted, may act on neighboring cells or extracellular phospholipids to augment lipid mediator biosynthesis, this idea has yet to gain traction because in vivo evidence is largely lacking. Our study provides to our knowledge the first clear in vivo evidence that sPLA2 acts in this...
manner, thus providing a rationale for the long-standing question on the role of the secreted type of PLA2. This extracellular PLA2 family, through a paracrine process, regulates homeostasis and pathology in response to a given microenvironmental cue. Given that PLA2G3 is insensitive to classical sPLA2 inhibitors, a new agent that specifically inhibits this unique sPLA2 may be useful for the treatment of patients with mast cell–associated allergic and other diseases.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession code.** Gene Expression Omnibus: GSE44980 (microarray data).

**Note:** Supplementary information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed the study, interpreted the findings and wrote the manuscript; N.U., T.K., Y.T. performed experiments and together with M.M. conceived and designed
**ONLINE METHODS**

**Mice.** *Pla2g3−/−, Pla2g4a−/−, Pla2g5−/−, Pla2g10−/−, Ptgdr−/−, Ptgdr2−/−, Ptgds−/−, Ptgds2−/−, Ptgdr−/−, Ptgdr2−/−, Ptgdr4−/−, Ptgdr−/−, Ptgdr4−/−, Ptgdr−/−, Tba2r−/−, Lib4r1−/−, Lib4r2−/−, Ltc4s−/−, AlocX5−/−* and *PLA2G3* mice have been described previously. The *Pla2g2e−/−* mice (RIKEN RBB04849) were generated by the Institute of Resource Development and Analysis (Kumamoto University). *Pla2gd2−/−, Pla2g2e−/−* and *Pla2g2f−/−* mice were generated by the Transgenic Resources Program (Department of Comparative Medicine, University of Washington; unpublished data). These mice were backcrossed to C57BL/6 mice for more than 11 generations, except for *Pla2g2f−/−* and *Ptgdr4−/−* mice (129Sv × C57BL/6), which were from the third backcross to C57BL/6 mice owing to severe problems in reproduction and neonatal death, respectively, particularly after successive backcrossing onto the C57BL/6 background. All experiments using knockout or transgenic mice (male, 8–12-week-old) were compared with their age-matched littermate control mice. Mast cell–deficient Kit− mutant mice, C57BL/6/J-KiW−/−Wt-wt and WBB6F1-KiW/−/−Wt-wt were purchased from the Jackson Laboratories and Japan SLC, respectively. All mice were housed in climate-controlled (23 °C) specific pathogen–free facilities with a 12-h light-dark cycle, with free access to standard laboratory food (CE2; CLEA) and water. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the Tokyo Metropolitan Institute of Medical Science and Showa University, in accordance with the Standards Relating to the Care and Management of Experimental Animals in Japan.

**PLA2 assay.** BV-PLA2 was purchased from Sigma-Aldrich. Recombinant mature human *PLA2G3* protein was expressed in silkworms by the baculovirus system and purified to near homogeneity by an affinity column conjugated with mouse monoclonal anti–human *PLA2G3* (SD2F1; IgG), as described previously. PLA2 activities in the supernatants of mast cells were assayed by measuring the amounts of [14C]linoleic acid released from 1-palmitoyl-2-[14C]linoleoyl-phosphatidylethanolamine (Perkin Elmer). Each reaction mixture consisted of appropriate amounts of the samples, 100 mM Tris-HCl (pH 7.4), 4 mM CaCl2, and the substrate at 1 μM. After incubation for 2 h at 37 °C, [14C]linoleic acid was extracted, and the radioactivity was quantified with a liquid scintillation counter (LS5801; Beckman), as described previously.

**Anaphylaxis.** For PSA, mice were intravenously injected with 3 μg of monoclonal anti-DNP IgE (SPE-7; Sigma-Aldrich). After 24 h, the mice were challenged intravenously with 25 or 500 μg of DNP-conjugated human serum albumin (HSA; Sigma-Aldrich) followed by incubation with 20 μg of chicken OVA (Sigma-Aldrich) or with 1 μg/ml anti-DNP IgE for 2 h and then stimulated with various concentrations (typically 100 ng/ml of DNP-BSA) of OVA (Sigma-Aldrich), 100 ng/ml mouse SCF or 10 ng/ml of OVA. Net ear swelling was measured for 6 weeks in MethoCult SFBIT (Iscove’s modified Dulbecco’s medium plus 10% FBS) to obtain BMDCs, BMDMs or BM cells. Colony stimulating factor (M-CSF; 100 ng/ml; Kyowa Kirin) for 3 d or mouse TSLP (1 μg/ml; R&D Systems) for 5 d in RPMI1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) to obtain BMDCs, BMDMs or BM basophils, respectively. The purity of each cell population was verified by flow cytometry for the expression of CD11c and MHC class II for BMDCs, the expression of F4/80 and CD11b for BMDMs, and the expression of FcεRI and CD200R3 (or DX5tr) coupled with a lack of expression of Kit for BM basophils (see below).

**Activation of BMMECs.** BMMECs (106 cells) before and after coculture with fibroblasts were sensitized with 1 μg/ml anti-DNP IgE for 2 h and then stimulated with various concentrations (typically 100 ng/ml of DNP-BSA) of OVA (Sigma-Aldrich), 100 ng/ml mouse SCF or 10 ng/ml of OVA (Sigma-Aldrich). Degranulation was evaluated by measuring histamine or β-HEX release, as described previously. Levels of eicosanoids were determined by ELISA in accordance with the manufacturer’s instructions (Cayman Chemical). Expression of cytokines was assessed by real-time PCR. Lactate dehydrogenase activity was measured using an LDH Cytotoxicity Detection Kit (Takara).

**Preparation and activation of mouse pMCs.** To collect peritoneal cells, 5 ml of Hank’s balanced salt solution (Invitrogen) was injected into the mouse peritoneal cavity, and the abdomen was massaged gently. After collecting peritoneal cells from the peritoneal fluid, they were resuspended in PIPES-buffered saline for electron microscopy or in BMMC-complete medium for degranulation assay. For degranulation, 106 cells were treated for 30 min with 1–5 μg/ml of human *PLA2G3* or BV-PLA2, with 1 μM A23187 (Sigma-Aldrich), or with 1 μg/ml anti-DNP IgE for 1 h and then with 100 ng/ml DNP-BSA for 30 min in the presence of 4 μM lypsothiadylserine (Avanti Polar Lipids). The release of histamine was then evaluated.

**Preparation of mouse skin mast cells and fibroblasts.** Mouse ear skin was dispersed with 1.6 mg/ml collagenase type II (Worthington) and 0.1 mg/ml DNease I (Sigma-Aldrich) in RPMI1640 containing 10% FBS for 30 min at 37 °C and passed through a cell strainer (40-μm mesh size). Skin mast cells were identified as Kit+FcεR1+ cells after CD45 gating by flow cytometry. The skin-dispersed cells were cultured in RPMI1640 containing 10% FBS, trypsinized and reseeded on culture dishes, and adherent cells grown to confluency were used as skin fibroblasts.

**Preparation and preparation of human mast cells and fibroblasts.** Preparation and culture of mast cells from human skin and lung were performed as described previously. Briefly, macroscopically normal human lung or skin tissue was obtained during surgery at the NIH University Hospital under approval of the faculty ethics committee and informed consent from the patient. Lung and skin cells were dispersed from chopped lung and foreskin specimens by collagenase and hyaluronidase (Sigma-Aldrich). These cells were maintained for 6 weeks in MethoCult SF+ (Iscorev’s modified Dulbecco’s medium (IMDM)-based serum-free medium containing 1.2% (w/v) methylcellulose;
Veritas) supplemented with human SCF (100 ng/ml; Peprotech) and human IL-6 (50 ng/ml; Peprotech). On day 42, methylnuclease was dissolled in PBS, and the cells were resuspended and cultured in IMDM medium (Invitrogen) containing 0.1% BSA, 100 ng/ml SCF and 50 ng/ml IL-6. The purity of human mast cells, as assessed with metachromatic staining, was more than 97%. Human skin and lung fibroblasts (CC-2511 and CC-2512, respectively) and their culture medium were purchased from Lonza. Human lung mast cells (5 × 10^5 cells) were seeded onto the human lung fibroblast monolayer and cocultured for 4 d in 500 µl of IMDM medium plus 2% FBS in the presence of SCF and IL-6, with medium change at 2-d intervals. The cells were trypsinized and reseeded in culture dishes, and nonadherent cells were collected. The purity of mast cells was normalized based on the expression of KIT.

**Real-time PCR.** Total RNA was extracted from tissues and cells using TRizol reagent (Invitrogen). First-strand cDNA synthesis was performed with the High-Capacity cDNA Reverse-Transcriptase Kit (Applied Biosystems). PCRs were carried out using the TaqMan Gene Expression System (Applied Biosystems) on an ABI7700 Real-Time PCR system (Applied Biosystems). The probe-primer sets are listed in **Supplementary Table 2**.

**Measurement of intracellular Ca^{2+} levels.** Intracellular Ca^{2+} levels were measured as described previously. Briefly, IgE-sensitized BMMCs on coverslips were loaded for 60 min with the fluorescent Ca^{2+} indicator fura-2/AM (5 µM; Invitrogen) in Tyrode-HEPES buffer (pH 7.4) containing 2.5 mM probenecid, 0.04% (v/v) pluronic acid and 1% (v/v) serum. Then, the cells were washed and stimulated with antigen. Fura-2 fluorescence images were obtained using an inverted microscope (Nikon Instruments) equipped with a cooled charge-coupled device camera (Hamamatsu Photonics) with excitation at 340 nm (F340) and 380 nm (F380) at 5-s intervals. The fluorescence ratio (F340/F380) was calculated using US National Institutes of Health Image J software.

**Western blotting.** Tissue homogenates (20 µg protein equivalent) or BMMCs (2 × 10^5 cells) were lysed in SDS-PAGE sample buffer (63 mM Tris-HCl (pH 6.8), 2% (v/v) SDS, 10% (v/v) glycerol, and 0.08% (w/v) bromophenol blue) containing 5% (v/v) 2-mercaptoethanol, and then subjected to SDS-PAGE. Proteins were subsequently blotted onto PVDF membranes (Bio-Rad), followed by blocking with 5% (w/v) milk powder in PBS containing 0.05% (v/v) Tween 20 (PBS-T). The membranes were probed with rabbit polyclonal antibodies to HDC, H-PGDS, Kit (1:500–1:2000) in the same buffer and then visualized with ECL Prime western blotting detection reagent (GE Healthcare Life Sciences) according to the manufacturer's instructions. After 48 h, wild-type BMMCs were cocultured for 2 d with the transfected cells.

**Adoptive transfer of BMMCs into mast cell–deficient mice.** BMMCs obtained from 8–12-week-old male mice were reconstituted by intratracheal (10^6 cells) or intravenous (10^5 cells) injection into 6-week-old male Kit<sup>W-sh/-sh</sup> mice. Six weeks after intradermal transfer or 12 weeks after intravenous transfer of BMMCs, the mice were subjected to IgE–Ag–induced PCA, as described above. Alternatively, mast cells from the base to the tip of the ears from these mice were evaluated by toluidine blue staining or by real-time PCR of mast cell marker genes.

**Flow cytometry.** Cells were stained with either a labeled monoclonal antibody or an isotype-matched control antibody (hamster IgG (HTK888), mouse IgG<sub>1</sub>, MOPC-21, rat IgG<sub>2</sub>, RTK2758 or rat IgG<sub>2</sub>, RTK4530; BioLegend) and analyzed by flow cytometry using FACS Calibur (BD Biosciences) or FACSaria III Cell Sorter (BD Biosciences) with FlowJo software (TreeStar). The antibodies used were specific for mouse Kit (2B8; BD Biosciences), FcR<sub>RII</sub> (MAR-1; eBioscience), DX5 (DX5; eBioscience), CD200R3 (Ba13; BioLegend), CD45 (30-F11; eBioscience), CD11c (N418; eBioscience), MHC class II (M5/114.15.2; eBioscience), CD11b (RA3-6B2; BD Biosciences), CD45R/B220 (RA3-6B2; BD Biosciences), CD45R/B220 (RA3-6B2; BD Biosciences), CD3e (145–2X11; eBioscience), Gr-1 (RB-8C5; BioLegend), FOXP3 (150D; BioLegend) and FR4 (12A5; BioLegend).

**Microarray.** Total RNA was extracted from BMMCs derived from Pla2g3<sup>−/−</sup> and Pla2g3<sup>+/−</sup> mice before and after coculture and purified using the RNeasy Mini Kit (Qiagen). The quality of RNA was assessed with a 2100 Bioanalyzer (Agilent Technologies). Both CDNA and cRNA were synthesized with a Low Input Quick Amp Labeling Kit according to the manufacturer's protocol (Agilent Technologies). Samples were hybridized to the Whole Mouse Genome Microarray Kit (4 × 44K; Agilent Technologies), washed and then scanned using a Laser Scanner GenePix 4000B (Molecular Devices). Microarray data were analyzed with Feature Extraction software (Agilent Technologies) and then imported into GeneSpringGX11.5 (Agilent Technologies). Probes were normalized by quantile normalization among all microarray data.

**RNA interference.** Swiss 3T3 fibroblasts were cultured in 12-well plates to 50% confluence and transfected with a Mission predesigned siRNA construct (20 nM) for Ptdga (SASI_Mm01_00116073 or 00116081; Sigma-Aldrich) or a scrambled control siRNA (Invitrogen) using oligofectamine (Invitrogen), according to the manufacturer's instructions. After 48 h, wild-type BMMCs were cocultured for 2 d with the transfected cells.

**Electrospray ionization mass spectrometry (ESI-MS).** ESI-MS lipidomics analysis using a 4000Q TRAP quadrupole-linear ion trap hybrid mass spectrometer (AB SCIEX) with an Ultimate 3000 nano/cap/micro-liquid chromatography system (Dionex Corporation) combined with an HTS PAL autosampler (CTC Analytics AG) was performed as described previously. Briefly, phospholipids extracted from 10<sup>7</sup> BMMCs were subjected directly to ESI-MS analysis by flow injection; typically, 3 µl of sample was applied. The mobile phase composition was acetonitrile/methanol/water (v/v/v = 6/7/2) plus 0.1% (v/v) ammonium formate (pH 6.8) at a flow rate of 10 µl/min. The scan range of the instrument was set at m/z 200–1,000 at a scan speed of 1,000 Da/s. The trap fill-time was set at 3 ms in the positive ion mode and at 5 ms in the negative ion mode. The ion spray voltage was set at 5,500 V in the positive ion mode and at −4,500 V in the negative ion mode.
mode. Nitrogen was used as a curtain gas (at a setting of 10 arbitrary units) and as a collision gas (set to 'high').

Statistical analysis. The Excel Statistical Program File ystat 2008 (Igaku Tosho Shuppan) was used to determine statistical significance evaluated by an unpaired Student’s t-test for two groups or an analysis of variance (ANOVA) for multiple groups. P values of less than 0.05 and 0.01 were considered statistically significant. Data are presented as the mean ± s.e.m.
