Dual Roles of Group IID Phospholipase A₂ in Inflammation and Cancer*  

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Phospholipase A₂ enzymes have long been implicated in the promotion of inflammation by mobilizing pro-inflammatory lipid mediators, yet recent evidence suggests that they also contribute to anti-inflammatory or pro-resolving programs. Group IID-secreted phospholipase A₂ (sPLA₂-IIId) is abundantly expressed in dendritic cells in lymphoid tissues and resolves the Th1 immune response by controlling the steady-state levels of anti-inflammatory lipids such as docosahexaenoic acid and its metabolites. Here, we show that psoriasis and contact dermatitis were exacerbated in Pla2g2d-null mice, whereas they were ameliorated in Pla2g2d-overexpressing transgenic mice, relative to littermate wild-type mice. These phenotypes were associated with concomitant alterations in the tissue levels of ω3 polyunsaturated fatty acid (PUFA) metabolites, which had the capacity to reduce the expression of pro-inflammatory and Th1/Th17-type cytokines in dendritic cells or lymph node cells. In the context of cancer, however, Pla2g2d deficiency resulted in marked attenuation of skin carcinogenesis, likely because of the augmented anti-tumor immunity. Altogether, these results underscore a general role of sPLA₂-IIId as an immunosuppressive sPLA₂ that allows the microenvironmental lipid balance toward an anti-inflammatory state, exerting beneficial or detrimental impact depending upon distinct pathophysiological contexts in inflammation and cancer.

Endogenous mechanisms that orchestrate the resolution of inflammation are essential for tissue homeostasis. When the resolution pathways are defective, acute inflammation can progress to chronic inflammation linked to fibrosis, metabolic diseases, and cancer (1–3). Lipid mediators derived from arachidonic acid (AA),2 including prostaglandins (PGs) and leukotrienes, are well known for their promoting roles in various inflammatory diseases, although they can also suppress immune responses under certain conditions (4, 5). Resolution of inflammation is also governed by specialized anti-inflammatory or pro-resolving lipid mediators, such as ω6 AA-derived lipoxins and ω3 polyunsaturated fatty acid (PUFA)-derived resolvins, protectins, and maresins (6, 7). Uncontrolled anti-inflammatory lipid programs perturb migration, clearance, polarization, and functions of neutrophils, macrophages, dendritic cells (DCs), and lymphocytes, leading to exacerbation of acute and chronic inflammation (8–14).

Phospholipase A₂ (PLA₂) is a group of lipolytic enzymes that hydrolyze the sn-2 position of glycerophospholipids to yield fatty acids and lysophospholipids. It has been established that group IVA cytosolic PLA₂ (cPLA₂)α plays a central role in releasing ω6 AA toward eicosanoid generation (15–17). The secreted PLA₂ (sPLA₂) family comprises the largest PLA₂ subfamily with 10 catalytically active isoforms, each of which displays distinct tissue distribution and substrate selectivity (18–20). Although sPLA₂s have been implicated in inflammation over the last few decades, their precise biological roles have remained enigmatic until recently. Recent studies using gene-manipulated mice for sPLA₂s have revealed that they display pro- or anti-inflammatory functions or even inflammation-unrelated functions by driving unique lipid pathways in response to specific extracellular microenvironmental cues (12, 14, 21–27). The mobilization of distinct lipids by sPLA₂s appears to rely not only on their intrinsic enzymatic properties, but also tissue- or disease-specific contexts such as the lipid composition of target membranes or the spatial and temporal availability of downstream lipid-editing enzymes (18), which may account for why sPLA₂s exert pro- or anti-inflammatory functions with different lipid mediator profiles in distinct settings.

Group IID sPLA₂ (sPLA₂-IIId) is preferentially and abundantly expressed in DCs in lymphoid organs (12), suggesting its regulatory role in acquired immunity. Indeed, a recent study

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1 The abbreviations used are: AA, arachidonic acid; DC, dendritic cell; BMDC, bone marrow-derived DC; CHS, contact hypersensitivity; DHA, docosahexaenoic acid; DMBA, 9,10-dimethylbenz(a)anthracene; DNBS, dinitrobenzene sulfonic acid; DNFB, dinitrofluorobenzene; EPA, eicosapentaenoic acid; ESI-MS, electrospray ionization mass spectrometry; IMQ, imiquimod; LN, lymph node; PLA₂, phospholipase A₂; sPLA₂, secreted PLA₂; cPLA₂α, cytosolic PLA₂α; PG, prostaglandin; Rv, resolvin; TG, transgenic; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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using Pla2g2d−/− mice have revealed that sPLA2-IID alleviates a Th1-driven immune response in the elicitation phase of contact hypersensitivity (CHS) by decreasing the level of ω3 PUFAs and their metabolites such as DHA-derived resolvin D1 (RvD1) in draining lymph nodes (LNs) (12). In addition, sPLA2-IID prevents an anti-virus Th1 immune response by directing the anti-inflammatory PGD2-DP1 (PGD2 receptor) axis, which prevents DC migration and thereby enhances viral infection and associated lung inflammation (28). Moreover, administration of sPLA2-IID-Fc protein to mice attenuates experimental autoimmune encephalomyelitis and colitis (29). To extend our understanding of the biological functions of sPLA2-IID, we have recently shown that, in the elicitation of the hapten antigen dinitrofluorobenzene (DNFB) to skin-regional LNs, a tissue in which sPLA2-IID is abundantly expressed. The marked decrease in the steady-state level of sPLA2-IID in acute inflammation remained unclear. We therefore examined the effect of Pla2g2d ablation on acute inflammation in the sensitization phase of CHS, which is equivalent to irritant dermatitis, as a model system.

Consistent with the view that Pla2g2d expression in DCs or macrophages is down-regulated after cell activation (12, 29), its steady-state expression in the LNs was very high (day −5, in accordance with the procedure of CHS; Fig. 1A) and markedly decreased 5 days after DNFB application (day 0) (Fig. 1A, bottom panel). Acute dermal edema (Fig. 1B) and migration of antigen-captured skin DCs to the regional LNs (12), which occurred within 24 h after DNFB treatment, did not differ between Pla2g2d+/+ and Pla2g2d−/− mice. However, we noticed that on day 0 (i.e. 5 days after DNFB treatment), LN expression of the inflammatory DC/macrophase markers Itgax (CD11c) and Itgam (CD11b) was significantly greater in Pla2g2d−/− mice than in Pla2g2d+/+ mice (Fig. 1C). When LN cells isolated from DNFB-treated mice were cultured ex vivo, Pla2g2d−/− cells produced more IFN-γ, a signature Th1 cytokine, than did Pla2g2d+/+ cells over 48 h (Fig. 1D). These results suggest that Pla2g2d deficiency results in exacerbation of the late stage of acute inflammation toward a Th1 response in skin-regional LNs, a tissue in which sPLA2-IID is abundantly expressed. Although Pla2g2d deficiency did not profoundly affect the increased (Ccl2 and Ccr5) or constitutive (Ccl19 and Ccr7) expression of DC/macrophase-attracting chemokines or chemokine receptors in the LNs on day 0, the expression of Crth2, which encodes the pro-inflammatory chemotactic PGD2 receptor CRTH2/DP2 (30, 31), was robustly elevated in DNFB-treated Pla2g2d−/− LNs over Pla2g2d+/+ LNs (Fig. 1E). The LN expression of Crth2 was much higher than that of Ptgsd2 (DP1), another PGD2 receptor that prevents DC migration (28, 32). In addition, the expression of Ptgsd2, a major PGD2 synthase in the LNs, was greater in Pla2g2d−/− mice than in Pla2g2d+/+ mice (Fig. 1E).

Electrospores ionization mass spectrometry (ESI-MS) analysis of lipids in the regional LNs under the steady-state condition (i.e. on day −5) revealed that the levels of ω3 PUFAs and their oxygenated metabolites were markedly reduced in Pla2g2d−/− mice relative to Pla2g2d+/+ mice (Fig. 1F). Although the level of ω6 AA was also lower in Pla2g2d−/− mice than in Pla2g2d+/+ mice, the levels of AA metabolites (i.e. PGs) were barely affected by Pla2g2d deficiency. These lipid profiles are consistent with our previous study and imply again that sPLA2-IID is preferentially coupled with the production of ω3 PUFA metabolites, whereas PGs are mainly produced from an sPLA2-IID-independent AA pool, most likely through the action of cPLA2α (12). After 5 days of DNFB application (day 0), however, differences in the LN levels of PUFAs between the genotypes became smaller (Fig. 1G), and the RvD1 level in Pla2g2d−/− LNs was markedly decreased to a level similar to that in Pla2g2d−/− LNs (Fig. 1H), being attributable, in part, to the down-regulation of Pla2g2d expression (Fig. 1A). Exclusively, the LN level of PGD2, but not PGE2, was greatly increased in Pla2g2d−/− mice relative to Pla2g2d+/+ mice on day 0 (Fig. 1G), corroborating the increased expression of Ptgsd2 in the null mice (Fig. 1E). Moreover, when LN cells from DNFB-treated mice on day 0 were stimulated with dinitrobenzene sulfonic acid (DNBS; a water-soluble form of DNFB) for 24 h ex vivo, PGD2 production, which depends on cPLA2α (15–17), was significantly greater in Pla2g2d−/− cells than in Pla2g2d+/+ cells (Fig. 1). These results suggest that the lack of sPLA2-IID exacerbates the late stage of LN inflammation in the process of irritant dermatitis with augmentation of the pro-inflammatory PGD2-CRTH2 axis and an immunological shift toward an increased Th1 immune response, which may be dependent on the steady-state reduction of ω3 PUFA metabolites.

Exacerbated Psoriasis in Pla2g2d−/− Mice—The marked decrease in the steady-state level of ω3 PUFA metabolites in Pla2g2d−/− LNs suggests that sPLA2-IID could also influence other types of immune response. To address this possibility, we next examined the effect of Pla2g2d ablation on psoriasis, a chronic inflammatory skin disease characterized by epidermal hyperplasia (acanthosis) due to aberrant proliferation and differentiation of keratinocytes, scaling, erythematous plaque formation, and increased production of pro-inflammatory (e.g. TNFα) and Th17-type (e.g. IL-17A, IL-22, and IL-23) cytokines. In a model of imiquimod (IMQ)-induced psoriasis (33), repeated IMQ challenges to mouse ears elicit psoriasis-like acanthosis and dermal swelling with Th17-type immune responses (a schema of the procedure is illustrated in Fig. 2A).

We found that IMQ-induced ear swelling and epidermal hyperplasia were significantly greater in Pla2g2d−/− mice than in Pla2g2d+/+ mice (Fig. 2, A–C). On day 6, the sPLA2-IID signal was colocalized in most, if not all, CD11c+ MHC-II+ der-
mal DCs in the affected skin of WT mice (Fig. 2D). Quantitative RT-PCR of the skin and draining LNs revealed that the expression levels of Th17-related cytokines (Il17a and Il22) and chemokine (Ccl20) were substantially higher in IMQ-treated Pla2g2d−/− mice than in Pla2g2d+/+ mice (Fig. 2, E and F), implying an increased Th17-type immune response in null mice. However, in contrast to sPLA2-IIID, an epidermal sPLA2 whose gene ablation leads to perturbed keratinocyte differentiation and activation (24), Pla2g2d deficiency did not affect the expression of the keratinocyte differentiation marker Krt14 (Fig. 2E). Consistent with the anti-inflammatory aspect of sPLA2-IIID (see above), LN expression of Pla2g2d in WT mice tended to decrease following IMQ treatment (Fig. 2F). Flow cytometry of IL-17A- or IL-22-expressing CD3ε+ T cells in the skin further supported the greater Th17 response in IMQ-treated and even IMQ-untreated Pla2g2d−/− mice than in replicate Pla2g2d+/+ mice (Fig. 2G). Lipidomics analysis of the affected skin revealed substantial reductions of DHA-derived metabolites such as RvD1 (7,8,17-trihydroxy-DHA) and RvD5 (7,17-dihydroxy-DHA) but not AA-derived eicosanoids such as PGE2 and PGD2 in Pla2g2d−/− mice, with the level of each lipid in Pla2g2d−/− mice as 1 (n = 5). G and H, ESI-MS analysis of PUFAs and their metabolites in the LNs of Pla2g2d−/− and Pla2g2d+/+ mice, with the level of each lipid in Pla2g2d−/− mice as 1 (n = 3). J, PGD2 production by LN cells that were obtained from DNFB-treated Pla2g2d+/+ and Pla2g2d−/− mice on day 0 and then stimulated with DNBS for 24 or 48 h ex vivo (n = 4). Values are mean ± S.E. *, p < 0.05, and **, p < 0.01.

**FIGURE 1. Increased LN inflammation in the late phase of irritant dermatitis in Pla2g2d−/− mice.** A, schematic representation of the procedure (top panel) and the LN expression of Pla2g2d, with the expression on day −5 being regarded as 1 (n = 4–5) (bottom panel), in the CHS model. The sensitization phase (irritant dermatitis) was analyzed in this study. The elicitation phase was detailed in our previous report (12). B, time course of ear swelling in Pla2g2d+/+ and Pla2g2d−/− mice with or without a single application of DNFB (n = 6). C, quantitative RT-PCR of Ilgax and Itgam in the draining LNs of Pla2g2d+/+ and Pla2g2d−/− mice treated for the indicated periods with DNFB, with Rn18s as a normalization control (n = 3–4). D, IFN-γ production by LN cells that were obtained from DNFB-treated Pla2g2d+/+ and Pla2g2d−/− mice on day 0 and then stimulated with DNBS for 24 or 48 h ex vivo (n = 4). E, quantitative RT-PCR of genes related to chemokines and lipid mediators in the LNs of Pla2g2d+/+ and Pla2g2d−/− mice, with Rn18s as a normalization control (n = 3–6). F, ESI-MS analysis of the steady-state levels of PUFAs and their metabolites in the LNs of Pla2g2d+/+ and Pla2g2d−/− mice, with the level of each lipid in Pla2g2d−/− mice as 1 (n = 5). G and H, ESI-MS analysis of PUFAs and their metabolites in the LNs of Pla2g2d+/+ and Pla2g2d−/− mice after treatment with DNFB for 5 days (G) or for the indicated periods (H) (n = 3–5). J, PGD2 production by LN cells that were obtained from DNFB-treated Pla2g2d+/+ and Pla2g2d−/− mice on day 0 and then treated with or without DNBS for 24 h ex vivo (n = 4). Values are mean ± S.E. *, p < 0.05, and **, p < 0.01.
Exacerbation of IMQ-induced psoriasis in Pla2g2d−/− mice. A, schematic representation of the procedure (top panel) and the kinetics of ear swelling in Pla2g2d−/+ and Pla2g2d−/− mice with or without IMQ treatment for the indicated periods (n = 20) (bottom panel). B, ear histology in Pla2g2d−/+ and Pla2g2d−/− mice with or without IMQ treatment for 6 days. C, evaluations of the area (top panel) and thickness (bottom panel) of Pla2g2d−/+ and Pla2g2d−/− ears on day 6 (n = 5). D, confocal immunofluorescence of sPLA2-IID (red), DC markers (CD11c and MHC-II; green), and their merged images in IMQ-treated WT mice on day 6, with Gapdh as a normalization control (n = 3–4). E, flow cytometry of IL-17A- or IL-22-expressing T cells in the skin of Pla2g2d−/+ and Pla2g2d−/− mice on day 6 (n = 3), with representative FACS profiles (left). H, ESI-MS analysis of AA-derived PGs and DHA-derived Rvs in the skin of IMQ-treated Pla2g2d−/+ and Pla2g2d−/− mice on day 6 (n = 8). Values are mean ± S.E., *p < 0.05, and **p < 0.01. B and D, representative images in two independent experiments are shown.

We then subjected Pla2g2d−/− mice and littermate WT mice to the models of psoriasis (Fig. 4, A–F) and CHS (Fig. 4, G and H). In sharp contrast to Pla2g2d+/− mice (Fig. 2), IMQ-induced ear swelling was significantly ameliorated in Pla2g2d−/− mice compared with WT mice (Fig. 4A). In both skin and LNs, Pla2g2d expression was markedly higher in Pla2g2d−/− mice than in WT mice, irrespective of IMQ challenge (Fig. 4B). The LN expression of Il17a and Il-22 was substantially lower in IMQ-treated Pla2g2d−/− mice than in replicate WT mice (Fig. 4C). This observation was further supported by flow cytometry, where the IMQ-induced increase in IL-17A- or IL-22-expressing CD3ε+ T cells was significantly reduced in Pla2g2d−/−-TG skin relative to WT skin (Fig. 4, D and E). Furthermore, the skin level of RvD1 was substantially higher in IMQ-treated Pla2g2d−/− mice than in WT mice (Fig. 4F).

In the elicitation phase of CHS, Pla2g2d ablation results in delayed resolution of Th1-driven dermatitis (12). Conversely, DNPB-challenged Pla2g2d−/− mice displayed an earlier recovery from ear swelling than did replicate WT mice, although progression of the edema during the initial 2 days was comparable between the genotypes (Fig. 4G). Moreover, on day 5, skin expression of inflammatory markers, including Il17a, Il23a, Il27, Il12, Ifng, Tnf, Il10, S100a9, Itgam, and Itgax, was consistently lower in Pla2g2d−/− mice than in WT mice (Fig. 4H). These results fully recapitulate the CHS phenotypes observed in Pla2g2d−/− mice (12) and sub-
sPLA\(_2\)-IID, an Immunosuppressive sPLA\(_2\)

Effects of PUFAs and Their Metabolites on Th17-type Immunity in Primary Culture—

Next, we used bone marrow-derived DCs (BMDCs) and primary LN cells in ex vivo culture to assess the roles of sPLA\(_2\)-IID and its potential lipid products in the Th17-type immune response in the context of psoriasis. After a 6-h stimulation with IMQ, an agent that induces the psoriasis-like Th17 response (see above), expression of \(\text{Il6}\) and \(\text{Il23a}\) was induced more robustly in \(\text{Pla2g2d}^{-/-}\) DCs than in \(\text{Pla2g2d}^{+/+}\) DCs (Fig. 5A). Additionally, \(\text{Pla2g2d}^{-/-}\) DCs expressed more \(\text{Il12a}\) (a Th1 cytokine) than did \(\text{Pla2g2d}^{+/+}\) DCs at each time point regardless of IMQ treatment (Fig. 5A), suggesting that sPLA\(_2\)-IID alleviates the Th1 immunity even under the basal state.

When WT-derived BMDCs were stimulated with IMQ in the presence of various lipids, the expression of \(\text{Il6}\) and \(\text{Il23a}\) was reduced modestly by \(\omega3\) PUFAs and markedly by several \(\omega3\) eicosapentaenoic acid (EPA) metabolites, including 18-hydroxy-EPA, RvD1, RvD2 (7,16,17-trihydroxy-DHA), protectin D1 (10,17-dihydroxy-DHA), and 10-hydroxy-DHA, whereas AA metabolites, including several PGs, leukotriene \(\text{B}\(_\text{3}\)\), and lipoxin A\(_4\) (LXA\(_4\)) failed to affect their expression significantly (Fig. 5B). Although EPA-derived RvE1 (5,12,18-trihydroxy-EPA) had no significant effects on the expression of \(\text{Il6}\) and \(\text{Il23a}\) (Fig. 5B), it significantly increased that of \(\text{Il10}\), an anti-inflammatory cytokine, in BMDCs (Fig. 5C). Expression of the psoriasis-related cytokines \(\text{Tnf}\) and \(\text{Il22}\) in IMQ-treated LN cells was reduced to varying degrees by various AA, EPA, and DHA metabolites as well as by DHA itself, whereas AA and EPA modestly enhanced the expression of \(\text{Il22}\) (Fig. 5D). Furthermore, RvD1, rather than its precursor DHA, reduced the secretion of IL-17A protein by IMQ-stimulated LN cells (Fig. 5E). Thus, although the effects of various PUFAs and their metabolites were not entirely identical and appeared to depend on the experimental conditions or cellular sources employed, it is likely that several \(\omega3\) (and even \(\omega6\)) PUFA metabolites have the capacity to potently block the Th17-driven inflammatory response.

sPLA\(_2\)-IID Exacerbates Skin Cancer by Attenuating Anti-tumor Immunity—

The aforementioned results, together with those of our previous study (12), suggest that sPLA\(_2\)-IID attenuates various types of inflammatory response (LN inflammation in irritant dermatitis, Th1-driven CHS, and Th17-driven psoriasis) through the production of pro-resolving lipid metabolites. However, this beneficial immunosuppressive property of sPLA\(_2\)-IID is conversely disadvantageous in certain situations such as host defense, as demonstrated by the finding that sPLA\(_2\)-IID suppresses an anti-viral Th1 immune response, leading to increased coronavirus infection and thereby augmented lung inflammation (28). We therefore speculated that, as in the case of anti-viral immunity, sPLA\(_2\)-IID might prevent anti-tumor immunity, thereby promoting tumor development.

To this end, we applied a model of chemical carcinogenesis induced by 9,10-dimethylbenz(a)anthracene (DMBA) and 12\(\alpha\)-tetradecanoylphorbol-13-acetate (TPA) to \(\text{Pla2g2d}^{-/-}\) mice and littermate WT mice on a BALB/c background, a strain that is sensitive to this model (a schema of the procedure is...
sPLA$_2$-IID, an Immunosuppressive sPLA$_2$

depicted in Fig. 6A). At 24 weeks, Pla2g2d$^{-/-}$ mice were highly protected from the development of skin tumors (Fig. 6B). Tumor incidence and multiplicity over time were delayed (Fig. 6, C and D), and the average tumor volume at 24 weeks was apparently small (Fig. 6E) in Pla2g2d$^{-/-}$ mice relative to Pla2g2d$^{+/+}$ mice. Among the knock-out mouse lines for several sPLA$_2$s, the significant reduction of tumor development was evident in Pla2g2d$^{-/-}$ (tumor weight and number) and Pla2g2f$^{-/-}$ (tumor weight) mice but not in Pla2g2e$^{-/-}$, Pla2g5$^{-/-}$, and Pla2g10$^{-/-}$ mice (Fig. 6, F and G). The protection of Pla2g2f$^{-/-}$ mice against skin cancer was ascribed to decreased keratinocyte hyper-growth by the absence of epidermal sPLA$_2$-IID, as we have reported recently (24).

At 4 weeks, an early time point when papillomas were still not apparent, the DMBA/TPA-induced epidermal thickening was already milder in Pla2g2d$^{-/-}$ mice than in Pla2g2d$^{+/+}$ mice (Fig. 7, A and B). Notably, the expression of Arg1 and Cd206, markers for tumor-promoting M2-like macrophages (34), but not Cd68, a marker for tumor-suppressing M1-like macrophages, was substantially lower in Pla2g2d$^{-/-}$ skin than in Pla2g2d$^{+/+}$ skin at 4 weeks after DMBA/TPA treatment (Fig. 7C). Consistent with this, flow cytometry of splenic (Fig. 7D) and LN (data not shown) cells revealed a reduction of M2-like macrophages (CD11chCD206lo) in Pla2g2d$^{-/-}$ mice than in replicate Pla2g2d$^{+/+}$ mice even under the steady-state condition. Quantitative RT-PCR of the regional LNs further confirmed the lower expression of the M2-like macrophage marker Arg1 in Pla2g2d$^{-/-}$ mice than in Pla2g2d$^{+/+}$ mice in both control and disease settings (Fig. 7E). In addition, Cd8a$^+$ IFN-γ$^+$ T cells in the regional LNs tended to be more abundant in DMBA/TPA-treated Pla2g2d$^{-/-}$ mice than in replicate Pla2g2d$^{+/+}$ mice (Fig. 7F). Moreover, the steady-state levels of RvD2 and maresin 1, which are DHA-derived lipid mediators that promote M2
macrophage polarization (40–45), were reduced in the LNs of Pla2g2d/h11002/h11002 mice compared with Pla2g2d/h11001/h11001 mice (Fig. 7G).

This difference was not seen in the DMBA/TPA-treated group as the levels of these lipid mediators in Pla2g2d/h11001/h11001 mice were decreased to their levels in Pla2g2d/h11002/h11002 mice, most likely due to down-regulation of Pla2g2d expression (see above). These results suggest that the absence of sPLA2-IID results in augmentation of anti-tumor immunity by decreasing tumor-promoting M2-like macrophages and increasing tumor-suppressing M1-like macrophages and cytotoxic T cells.

We then applied Pla2g2d-TG mice (C57BL/6 background) to the DMBA/TPA-induced skin carcinogenesis model. Beyond the limitation that the C57BL/6 strain is relatively insensitive to this model, constitutive expression of Arg1 was elevated (Fig. 7H) and DNBA/TPA-induced expression of Ifng was dampened (Fig. 7I) in Pla2g2d-TG skin compared with WT skin. Thus, as opposed to Pla2g2d deficiency, its TG overexpression shifts the immune balance toward suppression of the anti-tumor immunity.

Discussion

Impairment of the counter-regulatory pathways against pro-inflammatory immune responses is often detrimental and results in various diseases associated with chronic inflammation. With regard to the mechanisms underlying the effect of ω3 PUFAs on resolution mediator networks, it has been considered that resolvins and protectins, a family of ω3 PUFA-derived local mediators with unique anti-inflammatory activities and temporal profiles, occupy a position between the early response to inflammatory challenge and the resolution pathways where the initiating programs signal the termination (7, 35). As our understanding of the pro-resolving lipid mediator pathways has increased, there has been an emerging interest in the roles of PLA2s that can supply ω3 PUFAs upstream of their biosynthetic conversion to pro-resolving lipid mediators. Our recent efforts to clarify the biological functions of various PLA2s using knockout and/or transgenic strategies in combination with comprehensive lipidomics approaches have shown that two particular
sPLA₂s in specific locations, i.e. sPLA₂-IID in lymphoid DCs and sPLA₂-X in the colorectal epithelium, do mobilize ω3 PUFAs in vivo, thereby protecting against delayed-type hypersensitivity and colitis, respectively (12, 14).

Our recent study has demonstrated that sPLA₂-IID, a DC-expressed “resolving sPLA₂,” retards the resolution of Th1-driven inflammation in the skin and draining LNs by preferentially mobilizing DHA-derived pro-resolving lipid mediators (12). To gain further insights into the function of sPLA₂-IID, we have herein extended our study to other disease models using Pla2g2d⁻/⁻ mice as well as newly generated Pla2g2d-TG mice and obtained evidence that sPLA₂-IID ameliorates various types of immune response in general, including LN inflammation in acute irritant dermatitis, Th1-dependent CHS, and Th17-dependent psoriasis. This function could be explained, at least partly, by the ability of sPLA₂-IID to mobilize ω3 PUFA metabolites that dampen the Th1- or Th17-type immune responses. The lack of sPLA₂-IID markedly reduces the steady-state levels of ω3 PUFA metabolites by this sPLA₂ may put a brake on the ongoing DC-driven acquired immunity.

In contrast to its suppressive roles in CHS and psoriasis, sPLA₂-IID accelerates the development of skin tumors, probably because this enzyme attenuates anti-tumor Th1 immunity. This is reminiscent of the role of sPLA₂-IID in host defense against viral infection, where the reduction of anti-viral Th1...
immunity through the action of this sPLA₂ increases the infection and eventually worsens pulmonary inflammation (28). Given that some ω3 PUFA metabolites facilitate M2 polarization of macrophages (36, 37), our observations suggesting that tumor-associated M2-like macrophages, which promote cancer progression by releasing a panel of factors that prevent the recruitment or functions of cytotoxic T and NK cells (34), are reduced in Pla2g2d−/− mice and conversely increased in Pla2g2d-TG mice also fit with the current view. Thus, the immunosuppressive function of sPLA₂-IID provides favorable or unfavorable outcomes in distinct disease settings, thereby protecting against inflammation and exacerbating infection and cancer.

Reportedly, ectopic administration of ω3 PUFA metabolites (38–40) or systemic overproduction of these lipids in mice transgenic for Fat-1 (an ω3 PUFA synthase in Caenorhabditis elegans) (41) confers protective effects against infection-based inflammation or cancer xenograft by facilitating phagocytotic clearance of hazardous materials by innate immune cells such as neutrophils and macrophages. Apart from this systemic effect of ω3 PUFAs, the spatiotemporal supply of ω3 PUFAs by sPLA₂-IID, which is expressed preferentially and constitutively in DCs in local niches (particularly those in lymph tissues), may exert a distinct impact on acquired immunity by suppressing the functions of DCs, T cells, or their interactions. Indeed, multiple ω3 PUFA metabolites could attenuate the production of cytokines crucial for Th1/Th17 responses (this study) and reduce the migration or activation of DCs (12, 13, 28) and the functions of T and B cells (42, 43). Although the molecular basis underlying the actions of individual ω3 PUFA metabolites still remains elusive, it is tempting to speculate that they may exert their specific functions by acting on distinct receptors or through other mechanisms in different target cells. In this regard, the proposed mechanistic actions of ω3 PUFA metabolites include specific signaling through lipid-sensing receptors (9, 10, 44, 45), attenuation of lipotoxicity-induced endoplasmic
reticulum stress (46), and an increase in membrane fluidity leading to alterations of transmembrane or intracellular signaling (47), among others. In fact, ω3 PUFAs themselves can block Th17 cytokine production by lamina propria lymphocytes in colitis (14) and inflammasome activation in obesity (48–50) through the fatty acid receptor GPR40 or GPR120.

Nonetheless, the possibility that sPLA₂-IID exerts its anti-inflammatory function through mobilization of other lipids, including certain ω6 PUFAs, was not tested in this study, cannot be fully ruled out. Indeed, sPLA₂-IID appears to be coupled with the AA-PGD₂-DPF₁ axis in the lung LNs during pulmonary viral infection (28). Also, some lysophospholipid species, such as lysophosphatidylserine, have anti-inflammatory effects on innate and acquired immune responses through specific receptors (51, 52). Whether the immunosuppressive function of sPLA₂-IID could be linked to the generation of these anti-inflammatory lipids under particular disease conditions remains to be elucidated and requires further investigations.

The dual roles of sPLA₂s in promoting or attenuating diseases may be dictated by the cells they are secreted from, the target membranes they act on, the types of lipid metabolites they generate, or when and how these lipid metabolites are linked to particular biological processes in cell-, tissue-, or disease-specific contexts. For instance, sPLA₂-IIA, a prototypic sPLA₂ isoform that is inducible by pro-inflammatory stimuli, amplifies sterile inflammation by hydrolyzing membranes in microparticles, particularly extracellular mitochondria (53), whereas it has a protective effect against infection-induced inflammation such as sepsis and pneumonia by degrading membranes in invading bacteria (54). sPLA₂-V, which is induced by Th2 cytokines or metabolic stress, promotes M2 macrophage polarization and Th2 immunity by altering the local balance of unsaturated versus saturated fatty acids or by facilitating phagocytosis of harmful components, thereby aggravating Th2-driven asthma and attenuating Th1/Th17-related anti-fungal defense, arthritis, and obesity (23, 26, 27, 55, 56). sPLA₂-X exacerbates asthma by mobilizing AA metabolites in the lung (25), while protecting against colitis by mobilizing ω3 PUFAs in the colon (14). From these standpoints, sPLA₂-IID represents another example of the “good” and “bad” faces of this extracellular lipolytic enzyme family, by spatiotemporally supplying ω3 PUFAs in local tissue microenvironments where DC-driven acquired immunity plays fundamental roles in inflammation or in host defense against infection and cancer.

Overall, our current studies point to potential prophylactic or therapeutic use of an agent that would specifically stabilize or inhibit sPLA₂-IID according to disease contexts. Nonetheless, targeting sPLA₂-IID, as well as other sPLA₂s in general, will be a challenge, because these enzymes appear to play highly selective and often opposite roles in specific organs and disease states.

Experimental Procedures

Mice—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 Laboratory Diet, CLEA, Japan) and water. Pla₂g2d<sup>-/-</sup>, Pla₂g2e<sup>-/-</sup>, Pla₂g2f<sup>-/-</sup>, Pla₂g5<sup>-/-</sup>, and Pla₂g10<sup>-/-</sup> mice and their littermate controls, backcrossed to C57BL/6 or BALB/c mice (Japan SLC) for more than 12 generations, were described previously (12, 14, 22–24). 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 in accordance with the Japanese Guide for the Care and Use of Laboratory Animals.

Generation of Pla₂g2d-TG Mice—The strategy for generation of sPLA₂-overexpressing TG mice has been reported previously (57). In brief, mouse Pla₂g2d cDNA was inserted into the EcoRI site (downstream of the CAG (cytomegalovirus immediate early enhancer-chicken β-actin hybrid) promoter) in the pCALNL5 vector (Fig. 3A) (58). The plasmid, containing the transgene downstream of a neomycin cassette (Neo<sup>+</sup>) with Lox<sup>P</sup> sites at both ends, was excised at the HindIII and Sall sites to produce a 6-kb CAG-LoxP-Neo<sup>-</sup>-LoxP-Pla₂g2d (LNL-Pla₂g2d) fragment. Then the DNA was injected into fertilized eggs. Genotyping was performed on genomic DNA from tail biopsies by PCR using the primer pairs 5<sup>`</sup>-TGGTATTTGT-GCTGTCCTCATCATTT-3<sup>`</sup> and 5<sup>`</sup>-GGCTTTCTTCCCCGT-CATGTGT-3<sup>`</sup> (Sigma Genosys), which amplified a 1,300-bp fragment specific for LNL-Pla₂g2d. The reaction was conducted at 95 °C for 10 s and then 35 cycles at 95 °C for 0 s and 65 °C for 1 min on an Applied Biosystems 9800 Fast Thermal Cycler (Applied Biosystems). The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis with ethidium bromide. Male founders were mated with female C57BL/6 mice to confirm germ line transmission by PCR genotyping, and those with successful germ line transmission (LNL-Pla₂g2d-TG) were then crossed with female CAG-Cre<sup>/+</sup> mice, which carry a Cre recombinase transgene under control of the CAG promoter (58). This step resulted in removal of the Neo<sup>+</sup> cassette from the LNL-Pla₂g2d transgene, thereby allowing activation of the Pla₂g2d transgene in the whole body of the offspring. Pla₂g2d-TG mice were inbred with C57BL/6 mice. Phenotypes that appeared in Pla₂g2d-TG mice, which carried the active Pla₂g2d transgene, but not in LNL-Pla₂g2d-TG mice, in which the Pla₂g2d transgene remained silent, were regarded as events caused by the overexpressed sPLA₂-IID.

Quantitative RT-PCR—Total RNA was extracted from tissues and cells using TRizol reagent (Invitrogen). First-strand cDNA synthesis was performed using a high capacity cDNA reverse transcriptase kit (Applied Biosystems). PCRs were carried out using a Power SYBR Green PCR system (Applied Biosystems) or a TaqMan Gene Expression System (Applied Biosystems) on the ABI7300 Quantitative PCR system (Applied Biosystems). The probe/primer sets used are listed in Table 1. Histological Analysis—Histological analysis was performed as described previously (12). In brief, mouse tissues were fixed with 100 mM phosphate buffer (pH 7.2) containing 4% (w/v) paraformaldehyde, embedded in paraffin, sectioned, mounted on glass slides, deparaffinized in xylene, and rehydrated in ethanol with increasing concentrations of water. Hematoxylin and eosin staining was performed on the 5-μm-thick cryosections, and the stained sections were analyzed using a BX61 microscope (Olympus). Epidermal layer thickness was measured using DP2-BSW software (Olympus).
TABLE 1
List of primers used in quantitative RT-PCR

| Gene | Taq man probe (Applied Biosystems) | Gene | Taq man probe (Applied Biosystems) |
|------|-----------------------------------|------|-----------------------------------|
| Arg1 | Mm00479598_m1                   | Npp | Mm04346455_m1                     |
| C4B6 | Mm00417434_m1                   | Il1 | Mm04069988_m1                     |
| C208 | Mm00485148_m1                   | P2 | Mm0478250_m1                      |
| Cx36 | Mm04261351_x1                   | Ppyr | Mm04305650_m1                   |
| Fkg | Mm01160134_m1                   | Ppyr1 | Mm03306633_m1                   |
| F6 | Mm00464190_m1                   | Ppyr2 | Mm06873547_m1                   |
| F7a | Mm04396161_m1                   | Try | Mm0434260_g1                     |
| Rr22 | Mm0444241_m1                   | Gaps | Mouse GAPD (GAPDH), VIC/MGB, 4523398E |
| Rr23 | Mm01160011_g1                  | B2M | Eskyroic IFN-β mRNA, VIC/MGB, 435393E |

Confocal Laser Microscopy—Mouse frozen tissues, mounted in OCT compound (Sakura Finetek), were cut into 20-μm-thick sections using a cryomicrotome (CM3050S; Leica). The sections were incubated with 1.5× BlockAce® (DS Pharma Biomedical) in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (TBS) for 30 min and then with rabbit anti-mouse sPLA2-II antibody (50 μg/ml), phycoerythrin-labeled hamster anti-rabbit MHC class II antibody (M5/114.15.2; BioLegend), or FITC-labeled rat anti-mouse CD11c antibody (N418; eBioscience), or FITC-labeled hamster anti-mouse MHC class II antibody (MHC-II) antibody (55/114.15.2; BioLegend) in TBS containing 0.1% (w/v) bovine serum albumin (BSA) overnight at 4 °C. The sections were then treated with 1 μg/ml Alexa 647-labeled goat anti-rabbit IgG (Invitrogen) in TBS/BSA for 1 h, mounted in a Vectashield mounting medium (Vector Laboratories), and analyzed with a confocal laser scanning microscope (LSM510 META; Carl Zeiss). The laser scanning microscope (LSM510 META; Carl Zeiss) was used to examine the sections. The sections were incubated with 1.5 μg/ml Alexa 647-labeled goat anti-rabbit IgG (Invitrogen) in TBS/BSA for 1 h, mounted in a Vectashield mounting medium (Vector Laboratories), and analyzed with a confocal laser scanning microscope (LSM510 META; Carl Zeiss). The laser scanning microscope (LSM510 META; Carl Zeiss) was used to examine the sections.

Flow cytometry—Flow cytometry was performed as described previously (12, 24). Briefly, mouse inguinal LNs or spleen were excised and minced in Hanks’ solution (Nissui Pharmaceutical) with 2% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen) and 0.05% (w/v) sodium azide (Nakalai Tesque). Mouse ear skin was incubated with 0.25% (w/v) trypsin-EDTA (Sigma) for 60 min at 37 °C to separate the dermis from the epidermis. These tissues were then incubated with 400 units/ml collagenase type II (Worthington) with shaking for 30 min at 37 °C. After adding 10 mM EDTA, the resulting cell suspensions were passed through a 70-μm nylon cell strainer (Falcon™) and then centrifuged at 300 × g for 5 min at 4 °C. The isolated LN cells or splenocytes were treated for 2 min on ice with 10 mM Tris-HCl (pH 7.0) containing 0.84% (w/v) ammonium chloride to lyse red blood cells, centrifuged, and suspended in Hanks’ solution. The cells were blocked with mouse BD Tc Block™ (BD Biosciences), incubated with marker antibodies (listed in Table 2), and analyzed with a FACSAria III (BD Biosciences) and FlowJo (Tree Star) software.

Hapten-induced Contact Dermatitis—On day −5, mice (C57BL/6 background, 8–12-week-old males) were sensitized with 50 μl of 0.5% (w/v) DNFB (Sigma) in acetone/olive oil (4/1; v/v) on the shaved abdominal skin (sensitization phase). On day 0, the dorsal and ventral surfaces of the ears were challenged with 20 μl of 0.3% DNFB (elicitation phase). Ear thickness was monitored with a micrometer. To examine irritant dermatitis (corresponding to the sensitization phase of CHS), the ears were subjected to a single challenge with 50 μl of 0.5% DNFB. At the appropriate time points, the skin and regional LNs from the scarified mice were subjected to quantitative RT-PCR, histochemistry, flow cytometry, and lipidomics analysis, as described previously (12, 24).

IMQ-induced Psoriasis—Mice (C57BL/6 background, 8–12-week-old males) received a daily topical application of 12.5 μg of 5% (w/v) IMQ (Mochida Pharma) on the dorsal and ventral surfaces of the ears for 4 days (total 50 μg of IMQ cream per mouse). Ear thickness was monitored at various time points with a micrometer. On day 6, the skin and LNs from the scarified mice were subjected to quantitative RT-PCR, histochemistry, flow cytometry, and lipidomics analysis, as described previously (24).

Skin Carcinogenesis—The back skin of mice (BALB/c background, 8-week-old female) was shaved with an electric clipper. One week later, 200 μl of 2 mM DMBA (Sigma) in acetone was applied to the shaved skin. After 1 week, 200 μl of 80 μM TPA (Sigma) in acetone was applied to the skin twice a week over 24 weeks. Cutaneous papillomas were counted and scored weekly. At appropriate time points, the skin and LNs from the scarified mice were subjected to quantitative RT-PCR and histochemistry, as described previously (24). Flow cytometry of M1/M2-type macrophages and CD8+ T cells in the LNs was performed in accordance with the procedure described previously (60).

Cell Culture—Mouse LN cells were cultured in RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated FBS, 2 mM 1-glutamine, 25 mM Hepes, 50 μM 2-mercaptoethanol, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Bone marrow cells were cultured with mouse GM-CSF (10 ng/ml; PeproTech) for 9 days and with mouse M-CSF (100 ng/ml; Kyowa Kirin) for 3 days to obtain BMDCs and macrophages, respectively (61). The cells (2 × 106 cells/ml) were treated with or without 5 μg/ml IMQ or 100 μg/ml DNBS (Sigma) for appropriate periods. RNA extracted from the cells was taken for quantitative RT-PCR, and the supernatants were subjected to enzyme immunoassay for IFN-γ and IL-17A (eBioscience) or PGE2 (Cayman Chemicals). As required, the cells were pre-treated with 10 nM lipids (Cayman Chemicals) for 30 min and
then stimulated in 200 μl of serum-free X-VIVO medium (Lonza) in the continued presence of the lipids in 96-well U-bottom plates (Iwaki). These procedures were detailed in our previous study (12).

**ESI-MS**—Samples for ESI-MS of lipids were prepared and analyzed as described previously (12, 24). Briefly, tissues were soaked in 10 volumes of methanol and then homogenized with a Polytron homogenizer. After overnight incubation at −20 °C, water was added to the mixture to give a final methanol concentration of 10% (v/v). As an internal standard, 1 nmol of d_{35}-labeled EPA and d_{37}-labeled PGE_{2} (Cayman Chemicals) was added to each sample. The samples in 10% methanol were applied to Sep-Pak C18 cartridges (Waters), washed with 10 ml of hexane, eluted with 3 ml of methyl formate, dried up under nitrogen, and then stimulated in 200 l/s of serum-free X-VIVO medium. K. M. assisted Y. K. and M. S. M. M. M. wrote the manuscript with input from all the other authors.

**Author Contributions**—Y. M., Y. K., M. S., and K. Y. designed and performed the experiments. Y. T., C. T., and M. H. G. generated mutant mice. K. M. assisted Y. K. and M. S. M. M. wrote the manuscript with input from all the other authors.

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