Group V secretory PLA2 regulates TLR2-dependent eicosanoid generation in mouse mast cells through amplification of ERK and cPLA2α activation

Running Head: TLR2-dependent eicosanoid generation

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Abstract

Mast cells may be activated through Toll-like receptors (TLRs) for the dose- and time-dependent release of eicosanoids. However, the signaling mechanisms of TLR-dependent rapid eicosanoid generation are not known. We previously reported a role for group V secretory phospholipase A2 (PLA2) in regulating phagocytosis of zymosan and the ensuing eicosanoid generation in mouse resident peritoneal macrophages, suggesting a role for the enzyme in innate immunity. In the present study, we have used gene knockout mice to define an essential role for MyD88 and cytosolic PLA2α in TLR2-dependent eicosanoid generation. Furthermore, in mast cells lacking group V secretory PLA2, the time course of phosphorylation of ERK1/2 and of cPLA2α were markedly truncated leading to attenuation of eicosanoid generation in response to stimulation through TLR2, but not through c-kit or FceRI.

These findings provide the first dissection of the mechanisms of TLR-dependent rapid eicosanoid generation, which is MyD88-dependent, requires cPLA2α, and is amplified by group V sPLA2 through its regulation of the sequential phosphorylation and activation of ERK1/2 and cPLA2α. They support the suggestion that group V sPLA2 regulates innate immune responses.
**Introduction**

Mast cells, in addition to their role as central effector cells of allergic disease and various autoimmune and inflammatory processes, are important sensors of invading pathogens.\(^1\)\(^2\) The latter function is mediated through pattern recognition receptors that include CD48\(^3\) and the Toll-like receptors.\(^4\)\(^-\)\(^6\) Engagement of TLRs on human and mouse mast cells leads to robust generation of cytokines and chemokines.\(^4\)\(^-\)\(^{11}\) However, there are limited data on the generation of eicosanoids by mast cells in response to TLR signaling, and there has been no dissection of its mechanism.

Eicosanoids are generated from the polyunsaturated fatty acid, arachidonic acid, which is released from the sn-2 position of cell membrane phospholipids by one of the isoforms of phospholipase A\(_2\) (PLA\(_2\)). There are 4 main classes of PLA\(_2\), group IV cytosolic PLA\(_2\) (cPLA\(_2\)) secretory PLA\(_2\) (sPLA\(_2\)), group VI calcium-independent PLA\(_2\) (iPLA\(_2\)), and the acetyl hydrolases of platelet activating factor (groups VII and VIII).\(^12\) Mice in which the gene for cPLA\(_2\)\(_{\alpha}\) was disrupted were used to demonstrate its essential function in supplying arachidonic acid for eicosanoid biosynthesis in response to a wide range of stimuli.\(^13\)\(^,\)\(^14\) This leads to attenuation of allergic pulmonary inflammation\(^14\), acute lung injury in response to acid-aspiration\(^15\), cerebral infarction following ischemia/reperfusion injury\(^13\), and bleomycin-induce pulmonary fibrosis\(^16\), as well as difficulties with fertilization and parturition.\(^14\) The role of sPLA\(_2\) enzymes in eicosanoid generation has been inferred from transfection studies in which sPLA\(_2\) enzymes have been overexpressed and from studies using inhibitors of poor specificity. However, the natural role of the endogenous sPLA\(_2\) enzymes has remained unclear. To explore the role of endogenous group V sPLA\(_2\) we generated mice in which its gene was disrupted by homologous recombination. We previously reported that group V sPLA\(_2\) regulates the phagocytosis of the
yeast cell particle, zymosan \(^{17}\), and amplifies zymosan-induced eicosanoid generation from mouse resident peritoneal macrophages \(^{18}\), suggesting a role for this enzyme in innate immune responses.

We now demonstrate that TLR2-dependent LTC\(_4\) and PGD\(_2\) generation is attenuated in BMMC lacking group V sPLA\(_2\), further supporting a role for this enzyme in innate immunity. We show that TLR2-dependent eicosanoid generation by mouse BMMC requires cPLA\(_2\alpha\) and that this is a MyD88-dependent process. Group V sPLA\(_2\) amplifies the essential function of cPLA\(_2\alpha\) by regulating ERK-dependent cPLA\(_2\alpha\) phosphorylation in response to TLR2 stimulation.

**Materials and Methods**

**Materials**

The following reagents were purchased: the synthetic lipopeptide, S-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys\(_4\) (Pam\(_3\)CSK\(_4\)) (Invivogen, SanDiego, CA); peptidoglycan (PGN) from *Staphylococcus aureus* (Sigma, Saint Louis, MO); the mitogen activated protein kinase kinase (MEK) inhibitor, UO126 (Promega, Madison, WI); rabbit polyclonal antibodies that detect activating phosphorylations of extracellular signal-related kinase (ERK) 1 and ERK2 (Thr\(^{202}\)/Tyr\(^{204}\)), p-38 MAPK (Thr\(^{180}\)/Tyr\(^{182}\)), and cPLA\(_2\alpha\) (Ser\(^{505}\)) as well as the individual total proteins themselves (Cell signaling Technology, Danvers, MA); recombinant mouse SCF (Peprotech, Rocky Hill, NJ). Recombinant mouse IL-10 was expressed in baculovirus and its concentration was determined as described.\(^{19}\)

**Transgenic Mice**
Mice heterozygous for disruption of the gene encoding group V sPLA$_2$ ($Pla2g5$) were crossed for 11 generations to a BALB/cJ background. Mating pairs of $Pla2g5$-null and wild-type mice were derived from the litters of N11 BALB/cJ heterozygous breeding pairs. $Tlr2$-null and $Myd88$-null mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan). A homogenous population was established by backcrossing to C57BL/6 mice for more than ten generations. Mice with disruption of the gene encoding group IV cPLA$_2$$\alpha$ ($Pla2g4a$) were backcrossed to BALB/cJ mice for more than ten generations.

**Culture and activation of BMMC**

Mouse bone marrow cells were cultured in 50% enriched medium (RPMI 1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, 2mM L-glutamine, 0.1 mM non-essential amino acid, and 10% FBS) / 50% WEHI-3 cell (American Type Culture Collection, Manassas, VA) conditioned medium as described. After 4 weeks, >99% of cells were BMMC with characteristic metachromatic mast cell granules as assessed by staining with toluidine blue.

For TLR2-dependent mediator release, BMMC were washed and resuspended at 10$^6$ cells per 500 µl of culture medium and incubated with increasing doses of Pam$_3$CSK$_4$ for various periods. The reaction was stopped by centrifugation of the cells at 120 x g for 5 min at 4°C, and the supernatants were retained for assay of mediator release. In selected experiments, BMMC were cultured for 2 days in 50% WEHI-3 cell-conditioned medium containing 100 ng/ml SCF and 10 U/ml IL-10 prior to activation with PGN. PGD$_2$ and LTC$_4$ were assayed in the cell-free supernatant by EIA accordingly to the manufacturer’s instructions (LTC$_4$ - GE Healthcare, Piscataway, NJ; PGD$_2$ - Cayman Chemical, Ann Arbor, MI). Secretory granule exocytosis was determined by measuring the concentration of $\beta$-hexosaminidase in the supernatant and lysed cell
pellets as described. In selected experiments mast cells were sensitized for 1 h at 37°C with a 1:300 dilution of monoclonal IgE anti-TNP derived from ascites and stimulated with TNP-BSA or were stimulated with SCF as described.

**Arachidonic acid release**

Release of radiolabeled arachidonic acid was determined in BMMC that had been labeled to equilibrium by incubation overnight with $[^{14}C]$ arachidonic acid (0.1µCi [0.0037 MBq]/mL medium) and then washed prior to activation with Pam3CSK4. The amount of radiolabel released into the medium was expressed as a percent of intracellular $[^{14}C]$ lipids in unstimulated cells.

**Western Blotting**

BMMC (5 x 10^6 cells /ml) were activated with 3µg/ml of Pam3CSK4 for 1 to 60 min and the reaction was stopped by the addition of 2 volumes of ice-cold HBSS and centrifugation at 120 x g for 5 min at 4°C. The cell pellets were analyzed for expression of specific proteins by SDS-PAGE immunoblotting as described. Primary antibodies were used at 1 µg/ml. Bound primary antibodies were detected using goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3,000) (Jackson ImmunoResearch Laboratory, West Grove, PA) and enhanced chemiluminescence (Pierce, Rockford, IL). Membranes used for detection of phosphoproteins were stripped and reprobed with antibodies to the total (phosphorylated and non-phosphorylated) respective protein. The expression of each phosphoprotein was determined by densitometric analysis using ImageJ software. Expression was corrected for expression of total protein by dividing the intensity of each phosphoprotein band by the intensity of the respective total protein band.
Data analysis

All data are expressed as mean ± SEM and were analyzed by ANOVA for kinetic and time-course data. Statistical analysis for a single dose or time was performed using Student’s t test. Differences were considered statistically significant for p<0.05. In determining the “n” for any given experiment, each individual culture of BMMC was from a separate mouse and each “n” provides the number of separate BMMC cultures of each genotype from which data were derived.
Results

Group V sPLA₂ amplifies the cPLA₂α-dependent generation of eicosanoids by BMMC in response to TLR2 stimulation

We have previously reported that group V sPLA₂ amplifies the cPLA₂α-dependent generation of eicosanoids by resident peritoneal macrophages in response to zymosan\textsuperscript{18}, which signals in part through TLR2.\textsuperscript{26} To evaluate the role of group V sPLA₂ in the immediate phase of eicosanoid generation elicited in response to TLR2 signaling we measured LTC₄ and PGD₄ generation from \textit{Pla2g5}-null and wild type BMMC stimulated with the triacylated peptide Pam₃CSK₄, an agonist of the TLR1/TLR2 heterodimer. LTC₄ and PGD₂ were released in a dose- and time-dependent manner by both \textit{Pla2g5}-null and wild type BMMC that were maximal 60 min after stimulation with 3 µg/ml Pam₃CSK₄ (Figure 1). Neither eicosanoid was released at 60 min in the absence of Pam₃CSK₄ (Figure 1, C & D). The kinetics of eicosanoid generation appeared to be biphasic with a small initial release of LTC₄ and PGD₂ within the first 5 min and a second phase that began between 10 and 30 min and that was maximal at 60 min. There was no further increase in eicosanoid generation after 60 min (data not shown). The release of both eicosanoids was significantly attenuated in \textit{Pla2g5}-null BMMC compared to wild type BMMC, the attenuation being most marked at 60 min (p<0.001 and p<0.0001 for LTC₄ and PGD₂, respectively, by ANOVA for both dose-response and kinetic analyses). The maximal quantities of LTC₄ and PGD₂ generated in response to Pam₃CSK₄ were 449 ± 28 and 444 ± 43 pg/10⁶ cells, respectively, for wild type BMMC and 214 ± 22 and 156 ± 17 pg/10⁶ cells, respectively, for \textit{Pla2g5}-null BMMC. The release of eicosanoids in response to PGN, a TLR2/TLR6 agonist, was less robust than in response to Pam₃CSK₄, and was also attenuated in \textit{Pla2g5}-null BMMC (data not shown).
Eicosanoid generation from BMMC in response to PAM₃CSK₄ was not accompanied by release of the preformed granule-associated mediator, β-hexosaminidase (data not shown).

To directly determine the role of group V sPLA₂ in amplifying the release of arachidonic acid, we labeled BMMC to equilibrium with [¹⁴C]-labeled arachidonic acid and examined its release from Pla₂g₅-null and wild type BMMC in response to Pam₃CSK₄. Radiolabel was detected in the culture medium 60 min after stimulation. Net release was 0.31 ± 0.01 % and 0.16 ± 0.03 % in wild type and Pla₂g₅-null BMMC, respectively (n = 3; p = 0.01).

cPLA₂α is essential for the immediate phase of TLR2-dependent eicosanoid generation

To determine if cPLA₂α is essential for the immediate release of LTC₄ and PGD₂ by BMMC in response to TLR2 stimulation, BMMC from Pla₂g₄a-null and wild type control mice were stimulated with 0.1 to 3 µg/ml of Pam₃CSK₄ for 60 min (Figure 2A). LTC₄ and PGD₂ generation were undetectable in BMMC from Pla₂g₄a-null mice compared to 416 ± 55 pg/10⁶ cells of LTC₄ and 489 ± 62 pg of PGD₂/10⁶ cells, in control BMMC stimulated with 3 µg/ml of Pam₃CSK₄ (n=3; p<0.001).

cPLA₂α is activated by phosphorylation at serine 505 by the mitogen activated protein kinase (MAPK) extracellular receptor-stimulated kinase (ERK).²⁷ We therefore examined the effect of U0126, an inhibitor of MEK, the MAPK kinase immediately upstream of ERK, on eicosanoid generation and the phosphorylation of ERK and cPLA₂α. Wild-type BMMC were incubated with U0126 for 15 min prior to stimulation with 3 µg/ml PAM₃CSK₄ for 30 min. U0126 inhibited LTC₄ and PGD₂ generation from BALB/c BMMC in response to PAM₃CSK₄ in a dose-dependent manner (Figure 2B). Inhibition was apparent at doses greater than 1 µM and was complete with 5 µM U0126. Concentrations of the diluent, DMSO, equivalent to that
provided with 2 μM and 5 μM U0126, did not inhibit LTC₄ or PGD₂ generation. Phosphorylation of ERK1/2 and cPLA₂α were inhibited in a dose-dependent manner by U0126 (Figure 2C) confirming the role of ERK1/2 in the activation of cPLA₂α and the ensuing eicosanoid generation in response to TLR2 stimulation.

**Eicosanoid generation by BMMC in response to PAM₃CSK₄ requires TLR2 and MyD88**

To ensure that eicosanoid generation in response to Pam₃CSK₄ was dependent upon TLR2 and was not due to stimulation of an alternate receptor by an impurity in the preparation of the ligand, we examined eicosanoid generation by BMMC derived from Tlr₂-null mice and strain-matched C57BL/6 controls. In three separate experiments LTC₄ and PGD₂ generation in response to Pam₃CSK₄ were undetectable above background in Tlr₂-null BMMC (<25 pg/10⁶ cells) whereas wild-type C57BL/6 BMMC provided 725 ± 133 pg/10⁶ cells of LTC₄ and 1274 ± 586 pg/10⁶ cells of PGD₂ (Mean ± SEM, n=4). Eicosanoid generation in response to PGN was also absent in Tlr₂-null BMMC (data not shown).

TLRs signal through an adaptor protein that associates with their intracellular Toll/IL-1R/Resistance (TIR) domain. MyD88 is commonly used as the adaptor, and both MyD88-dependent and MyD88-independent pathways have been described.²⁸ We therefore examined the release of LTC₄ and PGD₂ in BMMC derived from Myd₈₈-null mice and strain-matched C57BL/6 controls. LTC₄ and PGD₂ generation in response to Pam₃CSK₄ were negligible and not significantly above background in Myd₈₈-null BMMC compared to that in C57BL/6 BMMC (p<0.002 and p< 0.001, respectively; n=3, Figure 3A).

In both Tlr₂-null and Myd₈₈-null BMMC there was detectable baseline phosphorylation of p42 ERK which did not increase in response to Pam₃CSK₄, in contrast to the time-dependent
and transient increase in p42 ERK phosphorylation that was observed in wild-type C57BL/6 BMMC (Figure 3B). Likewise the time-dependent sequential phosphorylation of p44 ERK and cPLA2α that were observed in wild-type C57BL/6 BMMC in response to Pam3CSK4 were not detectable in either Tlr2-null or Myd88-null BMMC (Figure 3B). Thus, TLR2 and MyD88 are essential for activation of ERK, phosphorylation of cPLA2α, and eicosanoid generation in response to Pam3CSK4 stimulation of BMMC.

**Group V sPLA2 amplifies the action of cPLA2 by regulating ERK phosphorylation**

To elucidate the mechanisms through which group V sPLA2 regulates cPLA2α-dependent eicosanoid generation, we examined the effect of deleting Pla2g5 on phosphorylation of cPLA2α and its upstream MAP kinases, ERK and p38. BMMC from Pla2g5-null and wild type mice were incubated with 3µg/ml of Pam3CSK4 for up to 60 min, and phosphoprotein expression was examined by Western blotting (Figure 4). The phosphorylation of cPLA2α in response to Pam3CSK4 was detectable within 1 to 2 min and increased to a maximum at 10 to 30 min in wild type BMMC; residual phosphorylation was still detectable 60 min after stimulation. In contrast, in Pla2g5-null BMMC phosphorylation was also detectable within 1 to 2 min, but the further rise in phosphorylation and sustained phosphorylation after 5 min were markedly attenuated (n = 3; p = 0.012; Figure 4 A & B).

Phosphorylation of ERK1/2 preceded that of cPLA2α and was apparent within 1 min of PAM3CSK4 stimulation in both wild type and Pla2g5 -/- BMMC (Figure 4 A, C & D). This phosphorylation was sustained to 10 min in wild-type BMMC and was barely detectable at 30 min. In contrast, ERK1/2 phosphorylation declined rapidly in Pla2g5 -/- BMMC and was undetectable by 10 min (n = 3; p = 0.015 compared to wild-type BMMC). There was no
phosphorylation of cPLA$_2$$\alpha$ or ERK1/2 up to 60 min in the absence of Pam$_3$CSK$_4$ (data not shown).

Phosphorylation of p38 MAP kinase was detectable in the absence of specific stimulation (Figure 4A) and increased modestly and to a similar extent in wild type and Pla$_2$g$_5$-null BMMC at 1 and 2 min after PAM$_3$CSK$_4$ stimulation.

The regulation of ERK and cPLA$_2$$\alpha$ by group V sPLA$_2$ is specific for TLR2 signaling

We have previously reported that the immediate phase of eicosanoid generation by BALB/c BMMC stimulated through FceRI or through c-kit is intact in Pla$_2$g$_5$-null BMMC$^{23}$. We therefore examined the phosphorylation of ERK and cPLA$_2$$\alpha$ in Pla$_2$g$_5$-null and wild type BALB/c BMMC in response to signaling through FceRI and c-kit. Pla$_2$g$_5$-null and wild-type BMMC were stimulated for 10 min with SCF or were sensitized with IgE anti-TNP and activated for 10 min with TNP-BSA. There was no difference in LTC$_4$ generation, or PGD$_2$ generation in response to IgE and antigen (Figure 5A) or SCF (Figure 5B) between Pla$_2$g$_5$ -/- and wild type BMMC, as previously reported. Phosphorylation of ERK1/2 was apparent within 1 min of stimulation, reached a maximum at 2 to 5 min, and then declined (Figure 5 C & D). The phosphorylation of cPLA$_2$$\alpha$ in response to SCF or TNP-BSA was detectable at 1 min and increased to a maximum at 10 to 30 min and then declined to baseline levels by 60 min (Figure 5 C & D). There was no detectable difference in the kinetics or degree of phosphorylation of cPLA$_2$$\alpha$ or ERK between wild type and Pla$_2$g$_5$-null BMMC in response to stimulation with IgE and antigen (Figure 5C) or SCF (Figure 5D).
Discussion

TLRs are dimeric receptors that recognize molecular signals on pathogenic organisms (pathogen associated molecular patterns). TLR2 dimerizes with TLR1 to recognize triacylated bacterial lipopeptides mimicked by PAM3CSK4 or with TLR6 to recognize diacylated peptides such as Mycoplasma macrophage activating lipopeptide 2 (MALP-2), PGN and lipoteichoic acid.\textsuperscript{28,29} In common with the IL-1\(\beta\) and IL-18 receptors, TLRs signal through a cytoplasmic TIR domain that associates with the adaptor protein MyD88, leading to activation of downstream kinases. MyD88-independent signaling can occur through TRIF (TIR domain containing adaptor inducing IFN\(\beta\)) or TRAM (TRIF-related adaptor molecule). These early signaling events couple to the NFkB pathway and to MAP kinases to regulate mediator release and cytokine secretion.

While there is a large body of data on the mechanisms of TLR-dependent cytokine generation, there is limited information on how TLRs elicit eicosanoid production. In general, eicosanoid biosynthesis can be divided into two phases. In the immediate phase, exemplified by FceRI-dependent mast cell activation, leukotriene and prostaglandin release begins within minutes of cellular activation and is dependent upon constitutively expressed 5-LO and COX-1, respectively\textsuperscript{19,30-32}. In the delayed phase, prostanoids are generated over several hours, without accompanying leukotriene biosynthesis, in a manner that requires induction of COX-2.\textsuperscript{19,32} LPS is well-established as a potent inducer of COX-2 expression and delayed prostanoid production.\textsuperscript{33} Use of siRNA and dominant negative constructs established a role for both TRIF and MyD88 in cPLA2\(\alpha\)-dependent release of arachidonic acid in the mouse RAW264.7 macrophage cell line in response to LPS.\textsuperscript{34} LPS-induced activation of cPLA2\(\alpha\) was also suppressed by inhibitors of p38 or ERK.\textsuperscript{34} While stimulation of human culture-derived mast cells with PGN elicited LTC\(4\) generation,\textsuperscript{5} the mechanism has not been explored. Our data
therefore provide the first insight into the mechanisms by which TLR signaling elicits the rapid generation of eicosanoids and specifically of leukotrienes.

LTC₄ and PGD₂ generation in response to TLR2 agonists was dependent upon cPLA₂α, which was activated by ERK (Figure 2). MyD88 was required for the sequential activation of ERK and cPLA₂α (Figure 3) suggesting that MyD88-independent pathways are not involved. While the early phases of ERK and cPLA₂α phosphorylation were intact in Pla2g5-null BMMC their sequential sustained phosphorylation was attenuated (Figure 4). Interestingly, eicosanoid generation by BMMC in response to TLR2 stimulation was biphasic with a small early phase that peaked within 10 min, and a second more substantial phase that was maximal at 60 min (Figure 1). The attenuation of PGD₂ and LTC₄ generation were most marked at 60 min. Our data therefore indicate that group V sPLA₂ regulates the activation of cPLA₂α by sustaining the activation of ERK, which is critical for the sustained activation of cPLA₂α in response to TLR2 stimulation. The sustained phosphorylation of cPLA₂α appears responsible for the later phase of LTC₄ and PGD₂ generation, which is therefore attenuated in Pla2g5-null BMMC.

sPLA₂ enzymes have previously been shown to stimulate the sequential activation of MAP kinase and cPLA₂α when added exogenously to mouse BMMC or the 1321N1 astocytoma cell line. Transfection of mouse mesangial cells with group V sPLA₂ or group IIA sPLA₂ enhanced H₂O₂-stimulated MAP kinase activation and cPLA₂α-dependent eicosanoid generation in an ERK-dependent manner. We provide the first demonstration for a role for endogenous group V sPLA₂ in regulating the sequential activation of ERK and cPLA₂α. It should be noted that we used BMMC derived from Pla2g5-null BALB/c mice. Because the allele carrying the disruption of Pla2g5 is derived from 129 ES cells, which carry a disruption of the neighboring Pla2g2a, these mice lack both group V sPLA₂ and group IIA sPLA₂.
group II A is not required for the sustained phosphorylation of ERK and cPLA2 is apparent from experiments with BMMC derived from C57BL/6 BMMC that also lack group II A sPLA2 and that were used as controls for mice deficient in TLR2, MyD88, or cPLA2α (Figure 3). Ongoing studies are addressing the mechanisms through which group V sPLA2 sustains the activation of ERK.

While exogenous sPLA2 or sPLA2 overexpressed by transfection in various cells is capable of amplifying the essential role of cPLA2α in the biosynthesis of eicosanoids, the role of the endogenous sPLA2 enzymes has remained poorly defined. Using mice in which the gene encoding group V sPLA2 was deleted, we demonstrated that this sPLA2 amplifies the essential function of cPLA2α in zymosan-induced LTC4 and PGE2 biosynthesis by resident mouse peritoneal macrophages. This translated in vivo to attenuation of the early phase of zymosan-induced peritonitis. We then demonstrated that group V sPLA2 regulates phagocytosis of zymosan by resident peritoneal macrophages. Others have demonstrated that group V sPLA2 has bactericidal activity toward *M. luteus* that is enhanced after treatment of the bacteria with lysozyme. These combined data suggest a role for group V sPLA2 in innate immune responses. Our present findings strengthen the suggestion that this sPLA2 plays a role in innate immunity, a role that is shared with group II A sPLA2, which is a more potent bactericidal agent than group V sPLA2 with a spectrum of activity that extends to gram negative bacteria. Furthermore, transgenic mice expressing human group II A sPLA2 are resistant to infection with *B. anthracis*, and in vivo administration of recombinant group II A sPLA2 protected mice against infection with this organism. Thus, a picture is emerging that sPLA2 enzymes may have evolved and retained their diversity in mammals due their role in innate immunity.
The role of group V sPLA2 in regulating eicosanoid generation, inflammation, and phagocytosis contrasts with the role of cPLA2α revealed through gene disruption. Lack of cPLA2α leads to profound deficits in the generation of leukotrienes and prostaglandins and results in the marked attenuation of inflammatory responses and also certain physiologic functions such as parturition \(^{13-16}\) raising important questions as to the possible side effects of targeting cPLA2α in human disease. In contrast, disruption of group V sPLA2 led to modest deficits in eicosanoid generation and phagocytosis.\(^{17,18,23}\) Thus, sPLA2 enzymes, and group V sPLA2 in particular, might be attractive therapeutic targets in inflammation, blunting rather than ablating the generation of lipid mediators. We have found that group V sPLA2 regulates eicosanoid generation from mouse BMMC in response to stimulation through TLR2 (Figure 1) with a limited role in regulating eicosanoid generation in response to signaling through FcεRI or c-kit that is limited to a strain-dependent role in delayed phase COX-2 induction and PGD\(_2\) generation (Figure 5).\(^{23}\) If this specificity extends to human cells, inhibition of group V sPLA2 might prove a useful strategy in targeting overexuberant TLR-dependent inflammation while leaving allergic inflammatory responses substantially intact. Whether targeting sPLA2 in humans would be accompanied by an increase in susceptibility to infection remains to be seen and will be addressed in the first instance in \textit{Pla2g5}-null mice. However, it is notable that, while mice lacking 5-LO display impaired phagocytosis and killing of \textit{K. pneumoniae},\(^{47}\) an increased risk of opportunistic infections from the use in humans of the 5-LO inhibitor zileuton has not emerged to date.

In conclusion our data provide the first dissection of rapid eicosanoid generation in response to TLR stimulation, demonstrate a role for endogenous group V sPLA2 in regulating the
sequential activation of ERK and cPLA$_2$$\alpha$, and provide further evidence that group V sPLA$_2$ is a participant in innate immune responses.
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EK designed and performed the research, collected and analyzed data, and wrote the paper. JVB contributed Pla2g4a-null mice. JPA designed the research, analyzed data, and wrote the paper.

None of the authors have any conflicts of interest.

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FIGURE LEGENDS

Figure 1. LTC₄ and PGD₂ generation in response to Pam₃CSK₄ are attenuated in Pla₂g₅-null BMMC. A & B. BMMC from wild type (shaded bars) and Pla₂g₅-null (open bars) mice, were incubated for 60min with the indicated concentrations of Pam₃CSK₄. C & D. BMMC from wild type (shaded bars) and Pla₂g₅-null (open bars) mice, were incubated for 0 to 60min with 3µg/ml of Pam₃CSK₄ or with medium alone (M) as a control. Supernatants were assayed for LTC₄ (A and C) and PGD₂ (B and D) by EIA. The values are the mean ± SEM of results 2 independent experiments obtained from six separate cultures of both wild type and Pla₂g₅-null BMMC (n=6). * p < 0.05, + p<0.01, ** p<0.001 by Student’s t test in post hoc analyses after ANOVA.

Figure 2. TLR2-dependent LTC₄ and PGD₂ generation in BMMC requires cPLA₂α. A. BMMC from wild type (shaded bars) and Pla₂g₄a-null (open bars) mice, were incubated for 60min with the indicated concentrations of Pam₃CSK₄. Supernatants were assayed for LTC₄ (left panel) and PGD₂ (right panel) by EIA. The values are the mean ± SEM of 2 independent experiments with three separate cultures of each strain of BMMC (n=3). B & C. BALB/c BMMC were treated with the indicated concentration of UO126 or with DMSO for 15 min and stimulated with (closed bars) or without (open bars) 3µg/ml Pam₃CSK₄ for 30 min. The supernatants were assayed for LTC₄ (left panel) and PGD₂ (right panel) by EIA (B); the values are the mean ± SEM (n=3). The phosphorylation of ERK-1/2 and cPLA₂α were assessed by Western blotting (C).
Figure 3. Activation of ERK and cPLA2α and the ensuing LTC4 and PGD2 generation by BMMC in response to Pam3CSK4 require TLR2 and MyD88. A. BMMC derived from Myd88−null (open bars) and wild type C57BL/6 mice (closed bars) were incubated for 60 min with the indicated concentrations of Pam3CSK4 for 60 min. The supernatants were assayed for LTC4 (left panel) and PGD2 (right panel) by EIA. (n=3). B. BMMC derived from Tlr2-null (n=3), Myd88−null (n=3) and wild type C57BL/6 mice (n=4) were incubated with 3 µg/ml Pam3CSK4 in a time-dependent manner. Pellets were analyzed for phosphorylated (left panels) or total (right panels) ERK and cPLA2α by Western blotting.

Figure 4. Group V sPLA2 amplifies ERK-dependent phosphorylation of cPLA2α. BMMC from wild type (left panels) and Pla2g5−null (right panels) mice, were incubated for 0 to 60 min with 3µg/ml of Pam3CSK4. Expression of phosphorylated (p) and total cPLA2α, ERK-1/2 and p38 was assessed by Western blotting. A. Representative data from 3 independent experiments are shown. B – D. Expression of phospho-cPLA2α (B), phospho-p44 ERK (C), and phospho-p42 ERK were quantified using the Image J software and were expressed as a ratio of the intensity of the respective total protein band (n=3).

Figure 5. The phosphorylation of ERK1/2 is intact in Pla2g5−null BMMC stimulated with IgE and antigen or SCF.

A & B. BMMC from wild type (shaded bars) and Pla2g5−null (open bars) mice, were sensitized with IgE anti-TNP and stimulated with 100 ng/ml TNP-BSA or buffer alone for 10 min (A) or were stimulated for 10 min with 100 ng/ml SCF or buffer alone (B). Supernatants were assayed for LTC4 (left) and PGD2 (right) by EIA. The values are the mean ± SEM of 2 independent
experiments with three separate cultures of each strain of BMMC (n=3). C & D. BMMC from
wild type (left) and Pla2g5–null (right) mice were stimulated with IgE and antigen (C) or with
SCF (D) for up to 60 min. Pellets were analyzed for phosphorylated (p) and total ERK-1/2 and
cPLA₂α by Western blotting.
Figure 1.

A  

B  

C  

D  

LTC₄ (pg/10⁶ cells)  

PGD₂ (pg/10⁶ cells)  

LTC₄ (pg/10⁶ cells)  

PGD₂ (pg/10⁶ cells)  

Pam₃CSK₄ (µg/ml)  

Pam₃CSK₄ (µg/ml)  

0 1 2 5 10 30 60 60  

(min)  

M  

M  

0 0.1 1 3  

0 0.1 1 3  

0 1 2 5 10 30 60 60  

0 1 2 5 10 30 60 60  

*  

**  

*  

+  

*  

+  

*  

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Figure 2

A.

B.

C.

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Figure 3

A. 

B. C57BL/6

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<td>cPLA₂α</td>
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TLR2-null

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MyD88-null

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Figure 4

A. Wild type  Pla2g5-null

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Time (min) 0 1 2 5 10 30 60

B. Densitiy (Phospho/Total)

C. Densitiy (Phospho/Total)

D. Densitiy (Phospho/Total)
Figure 5

A. IgE + Ag

- LTC4 (ng/10⁶ cells) vs. Time (min) without TNP-BSA
  - 0, 10, 30 min

B. SCF

- LTC4 (ng/10⁶ cells) vs. Time (min) without SCF
  - 0, 10, 30 min

C. IgE + Ag

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D. SCF

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Time (min) 0 1 2 5 10 30 60
Group V secretory PLA₂ regulates TLR2-dependent eicosanoid generation in mouse mast cells through amplification of ERK and cPLA₂α activation

Eriya Kikawada, Joseph V Bonventre and Jonathan P Arm

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