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Phospholipase C β 3 deficiency leads to macrophage hypersensitivity to apoptotic induction and reduction of atherosclerosis in mice

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Atherosclerosis is an inflammatory disease that is associated with monocyte recruitment and subsequent differentiation into lipid-laden macrophages at sites of arterial lesions, leading to the development of atherosclerotic plaques. PLC is a key member of signaling pathways initiated by G protein-coupled ligands in macrophages. However, the role of this enzyme in the regulation of macrophage function is not known. Here, we studied macrophages from mice lacking PLC β 2, PLC β 3, or both PLC isoforms and found that PLC β 3 is the major functional PLC β isoform in murine macrophages. Although PLC β 3 deficiency did not affect macrophage migration, adhesion, or phagocytosis, it resulted in macrophage hypersensitivity to multiple inducers of apoptosis. PLC β 3 appeared to regulate this sensitivity via PKC-dependent upregulation of Bcl-XL. The significance of PLC β signaling in vivo was examined using the apoE-deficient mouse model of atherosclerosis. Mice lacking both PLC β 3 and apoE exhibited fewer total macrophages and increased macrophage apoptosis in atherosclerotic lesions, as well as reduced atherosclerotic lesion size when compared with mice lacking only apoE. These results demonstrate what we believe to be a novel role for PLC activity in promoting macrophage survival in atherosclerotic plaques and identify PLC β 3 as a potential target for treatment of atherosclerosis.

Introduction

PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate 2 second messengers, inositol 1,3,4-triphosphate and diacylglycerol. While inositol 1,3,4-triphosphate regulates Ca^{2+} efflux, it and diacylglycerol activate PKC (1). The β family of PLC, which consists of 4 isoforms, PLC β 1– β 4, is regulated by heterotrimeric G proteins (2). PLC β 2 is primarily expressed in hematopoietic cells, whereas PLC β 3 and PLC β 1 are expressed in a wide range of cells and tissues (2). PLC β 4 is predominantly expressed in neuronal cells (3, 4). While all of the PLC β isoforms can be activated by the members of the Gq family of α subunits, PLC β 2 and β 3 can also be potentially activated by G $\beta\gamma$ (5).

Macrophages are a heterogeneous population of leukocytes present ubiquitously in various tissues of the body. They are derived from monocytes after monocytes leave blood vessels. The heterogeneity of macrophages is shaped by the diverse microenvironments in which these cells are found. Macrophages have a role in regulating a wide range of homeostatic, immunological, and inflammatory processes (6, 7). One of the important biological functions of macrophages is host defense against microbial infections. Macrophages perform this duty by nonspecific killing through cap-

turing and engulfing microbes and/or activating specific lymphocyte-based immune responses through antigen presentation and cytokine production. In addition to host defense, macrophages are inseparably associated with inflammation and particularly chronic inflammation, which is now believed to underlie many human diseases including atherosclerosis. Atherosclerosis is an inflammatory disease potentiated by overrecruitment of leukocytes – particularly monocytes/macrophages – into an arterial lesion (8–13). Oxidized LDL (oxLDL), among many possible triggers, has been identified as an initial inducer of endothelial cell and SMC dysfunction. These dysfunctional cells produce proinflammatory cytokines including chemokines, which, together with increased adhesiveness of endothelial cells, recruit monocytes to the intima. These monocytes then become macrophages, whose phagocytic activity allows the macrophages to become hyperlipidemic foam cells, the building blocks for atherosclerotic plaques. The foam cells eventually undergo apoptosis and produce more proinflammatory cytokines and chemokines that lead to recruitment of more monocytes. This process is one of the key pathogenic bases for the initiation and progression of atherosclerosis.

Studies using mice lacking monocyte chemoattractant protein 1 (MCP-1) or its receptor CCR2 provided strong genetic evidence for the involvement of chemokines in the pathogenesis of atherosclerosis (14, 15). When these mice were fed a high-fat diet (HFD), there was a decrease in atherosclerotic lesion size and a reduction in the number of infiltrated macrophages in the lesion (14, 15). This conclusion was further supported by a number of follow-up studies (16–21). In addition to MCP-1, other chemokines and

Nonstandard abbreviations used: CHX, cycloheximide; HFD, high-fat diet; LPA, lysophosphatic acid; MCP-1, monocyte chemoattractant protein 1; oxLDL, oxidized LDL; PAF, platelet-activating factor; SDF-1, stromal cell-derived factor 1; S1P, sphingosine 1-phosphate.

Conflict of interest: The authors have declared that no conflict of interest exists.

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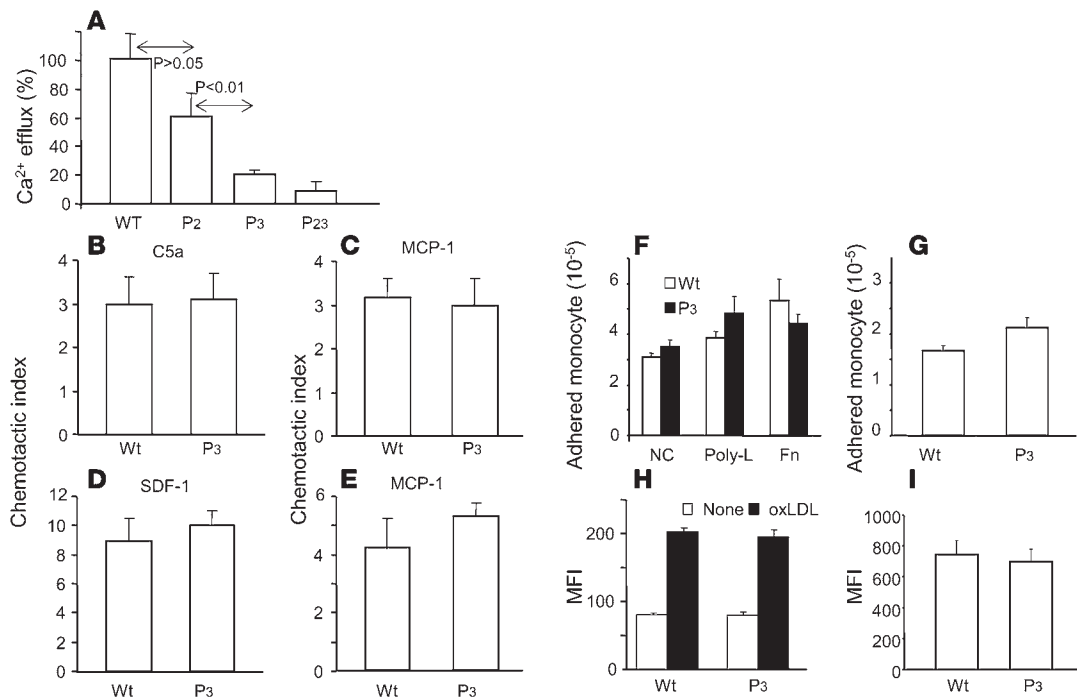


Figure 1

Effects of PLC $\beta 3$ deficiency on macrophage functions. (A) Calcium effluxes induced by 10 nM C5a in macrophages isolated from wild-type mice and mice lacking PLC $\beta 2$ (P₂), PLC $\beta 3$ (P₃), or both (P₂₃). (B–E) Transendothelial migration of peritoneal macrophages and spleen monocytes. Peritoneal macrophages (B and C) or spleenocytes (D and E) were added to the top chambers of Transwell plates precoated with mouse endothelial cells. C5a (10 nM), SDF-1 (100 ng/ml), or MCP-1 (60 ng/ml) were added to the lower chambers. After 4-hour incubation, cells in the lower chambers were collected, counted, and analyzed by FACS after staining with F4/80 (B and C) or Gr-1^{lo}CD11b^{mid} (D and E) antibodies. The indices were calculated by dividing the number of F4/80-positive (B and C) or Gr-1^{lo}CD11b^{mid} (D and E) cells in the presence of ligand by that in its absence. $n = 8$; $P > 0.05$ for all B–E. (F and G) Adhesion of spleen monocytes to extracellular matrices and endothelial cells. Spleen cells were added onto 24-well plates without coating or coated with poly-lysine, fibronectin (F), or mouse endothelial cells (G). After 10 minutes, attached cells were collected, counted, and analyzed by FACS for Gr-1^{lo}CD11b^{mid} monocytes. $n = 4$; $P > 0.05$ for F and G. (H) Macrophage phagocytosis. Peritoneal macrophages were seeded on 24-well culture plates. Uncoated and oxLDL-coated FluoSphere beads were added to the cells. After extensive washes, cells were detached, stained with F4/80, and analyzed by FACS. $n = 4$; $P > 0.05$ between genotypes. (I) Uptake of apoptotic Jurkat cells. Macrophages were incubated with CFDA SE-labeled apoptotic Jurkat cells, detached, stained with a macrophage marker F4/80, and analyzed by FACS. The MFI of CFDA SE associated with F4/80-positive cells was determined. $n = 4$; $P > 0.05$. fn, fibronectin.

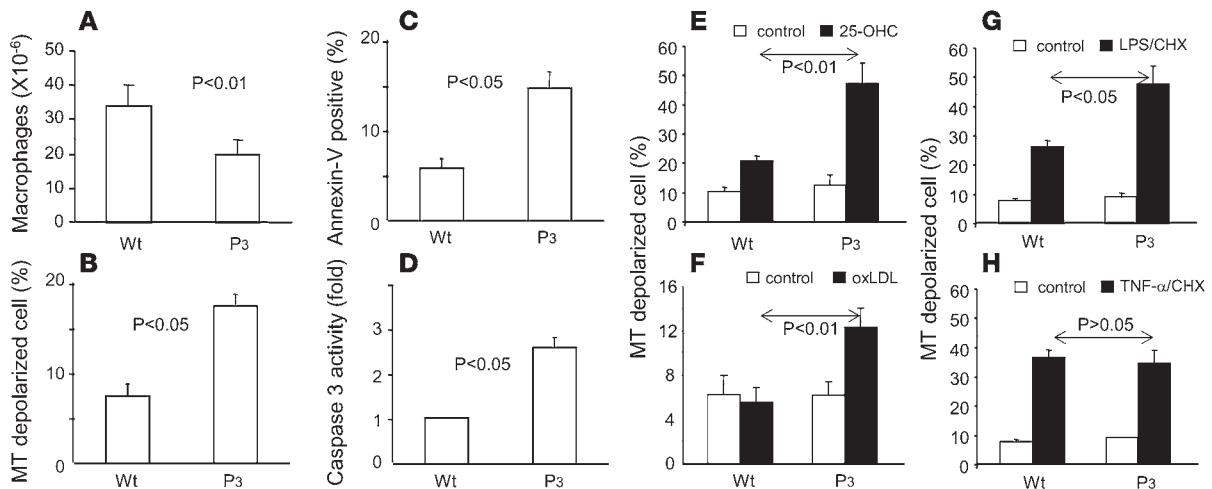
chemokine receptors have also been demonstrated to contribute to the initiation and progression of atherosclerotic lesion (9, 12). However, there is a lack of study of roles of signaling pathways activated by chemokines in atherogenesis, except for the recent study of PI3K γ (22). Chemokines bind to their specific G protein-coupled receptors and regulate a number of signaling pathways that include PLC, PI3K, MAPKs, the Rho family of small GTPases, and adenylyl cyclases (23–25).

We have previously generated mice lacking PLC $\beta 2$, PLC $\beta 3$, and both and found that chemokines use both PLC β isoforms in mouse neutrophils, even though PLC $\beta 2$ appeared to be the predominant isoform (26, 27). Although neutrophils lacking both PLC $\beta 2$ and PLC $\beta 3$ produced no Ca²⁺ efflux in response to fMLP or IL-8, potent neutrophil chemoattractants, no significant chemotactic defects were found with these neutrophils using Transwell assays (26, 27). This suggests that PLC signaling may be dispensable in cell migration. Macrophages, like neutrophils, also respond to certain chemokines (MCP-1 and stromal cell-derived factor 1 [SDF-1], for instance) and other G protein-coupled ligands (lysophosphatic acid [LPA] and sphingosine 1-phosphate [S1P], for instance) to produce Ca²⁺ efflux via activa-

tion of PLC. In this study, we investigate the role of PLC $\beta 2$ and PLC $\beta 3$ in G protein-coupled ligand-induced PLC activation and the role of PLC signaling in macrophage responses and in vivo functions.

Results

To investigate whether PLC $\beta 2$ or PLC $\beta 3$ is involved in chemoattractant-induced PLC activity in macrophages, we examined C5a-induced Ca²⁺ efflux in peritoneal macrophages isolated from wild-type, PLC $\beta 2$ -null, PLC $\beta 3$ -null, and PLC $\beta 2/\beta 3$ -double-null mice. As shown in Figure 1A and Supplemental Figure 1 (supplemental material available online with this article; doi:10.1172/JCI33139DS1), PLC $\beta 3$ deficiency led to a greater reduction in C5a response than PLC $\beta 2$ deficiency, and PLC $\beta 2/\beta 3$ double deficiency almost abolished C5a-induced Ca²⁺ efflux. We also measured responses to a number of other G protein-coupled ligands, including SDF-1, MCP-1, platelet-activating factor (PAF), LPA, and S1P. Similar results were observed (data not shown). These data together indicate that although both PLC $\beta 2$ and PLC $\beta 3$ are required for G protein-coupled ligand-induced PLC activation, PLC $\beta 3$ is the major functional isoform in these macrophages.

**Figure 2**

PLC $\beta 3$ deficiency leads to an increased sensitivity of macrophages to apoptosis induction. (A–D) Increased apoptosis in macrophages from the 3-day thioglycolate-elicited model. (A) The numbers of peritoneal macrophages recovered from mice injected with thioglycolate for 3 days were counted ($n = 27$). (B) Percentages of macrophages with depolarized mitochondria ($n = 4$) were determined by FACS with the Mito-Probe dye and the anti-F4/80 antibody. (C) Percentages of annexin V–positive macrophages ($n = 5$) were determined by FACS analysis with annexin V and F4/80 antibodies. (D) Caspase-3 activity ($n = 5$) was determined using a caspase-3 assay kit from Roche Inc. (E–H) Increased sensitivity to apoptotic induction in macrophages from the 1-day thioglycolate-elicited model. Mice were injected with thioglycolate for 1 day, and percentages of macrophages with depolarized mitochondria ($n > 4$) were determined by FACS. For treatments, cells were incubated with 10 $\mu\text{g/ml}$ 25-OHC, 100 $\mu\text{g/ml}$ oxLDL, LPS (1 $\mu\text{g/ml}$)/CHX (1 $\mu\text{g/ml}$), or TNF- α (10 ng/ml)/CHX (10 $\mu\text{g/ml}$) for 24 hours. MT, mitochondria.

Next, we investigated the significance of PLC $\beta 3$ -mediated signaling in regulation of cellular responses of macrophages and monocytes, the precursors of macrophages, which include migration, adhesion, phagocytosis, and apoptosis. The effects of PLC $\beta 3$ deficiency on macrophage chemotaxis in response to MCP-1 and C5a were examined using the Transwell migration assay. No significant effects were observed on macrophage migration through either a bare polycarbon membrane (data not shown) or mouse endothelial cells (Figure 1, B and C). We also examined the migration of monocytes in response to SDF-1 and MCP-1, and no significant differences between wild-type and PLC $\beta 3$ -deficient spleen monocytes were observed in the transendothelial Transwell assay (Figure 1, D and E). We next examined the effects of PLC $\beta 3$ deficiency on monocyte adhesion. Spleen monocytes from wild-type and PLC $\beta 3$ -null mice showed the same ability to adhere to the uncoated surface or surfaces coated with poly-lysine or fibronectin (Figure 1F). These monocytes also showed the same adherence to mouse endothelial cells (Figure 1G). In addition, PLC $\beta 3$ deficiency did not appear to affect the ability of macrophages to uptake uncoated or oxLDL-coated FluoSphere beads (Figure 1H), nor did it affect the engulfment of anti-Fas antibody-induced apoptotic Jurkat cells (Figure 1I). Putting these results together, we believe that PLC $\beta 3$ deficiency does not appear to cause significant alterations in migration, adhesion, phagocytosis, or uptake of oxLDL or apoptotic cells.

When we isolated macrophages for the aforementioned functional studies from the peritonea of mice injected with thioglycolate for 3 days, we noticed that there was a statistically significant reduction in the number of macrophages from PLC $\beta 3$ -null mice compared with wild-type mice (Figure 2A). Given that there were no differences in macrophage/monocyte migration and adhesion, the difference in cell number suggests that PLC $\beta 3$ -mediated signaling may be involved in regulation of macrophage survival. We went

on examining whether there were more apoptotic macrophages in these preparations using annexin V and Mito-Probe staining. Mito-Probe dye produces stronger fluorescence when staining the normal mitochondria than the permeabilized ones that are often found in apoptotic cells (28), whereas annexin V stains phosphatidylserine in the outer leaflet of the plasma membranes of apoptotic cells. Significant increases in macrophage apoptosis in the preparations from PLC $\beta 3$ -null mice were detected by both staining methods (Figure 2, B and C, and Supplemental Figure 2, A–D). We also measured caspase-3 activity and found that PLC $\beta 3$ -deficient macrophages also had significantly higher caspase-3 activity than the wild-type cells (Figure 2D). All of these observations suggest that PLC $\beta 3$ signaling is involved in regulation of macrophage apoptosis. However, when macrophages isolated from mouse peritonea after 1-day thioglycolate elicitation were examined, we found no significant differences in macrophage apoptosis as measured by Mito-Probe (Figure 2E) or annexin V staining (data not shown). Together with the observations of no significant changes in either cell number or apoptosis of monocytes in the peripheral blood, spleen, and bone marrow (data not shown), we hypothesize that the lack of PLC $\beta 3$ may not normally affect macrophage/monocyte apoptosis; rather it may render the macrophages more sensitive to apoptotic inducers that may become more abundant in 3-day than 1-day thioglycolate-elicited peritonea.

To test this possibility, we examined sensitivity of 1-day thioglycolate-elicited peritoneal macrophages to a number of apoptosis inducers. Oxysterols, components of oxLDL, are potent inducers of macrophage apoptosis (29–31). Treatment of wild-type macrophages with 25-OHC, an oxysterol, resulted in an about 1-fold increase in the number of apoptotic macrophages, whereas treatment of PLC $\beta 3$ -null macrophages resulted in more than a 4-fold increase (Figure 2E). This hypersensitivity to 25-OHC-induced apoptosis could also be recapitulated in mouse macrophage-like

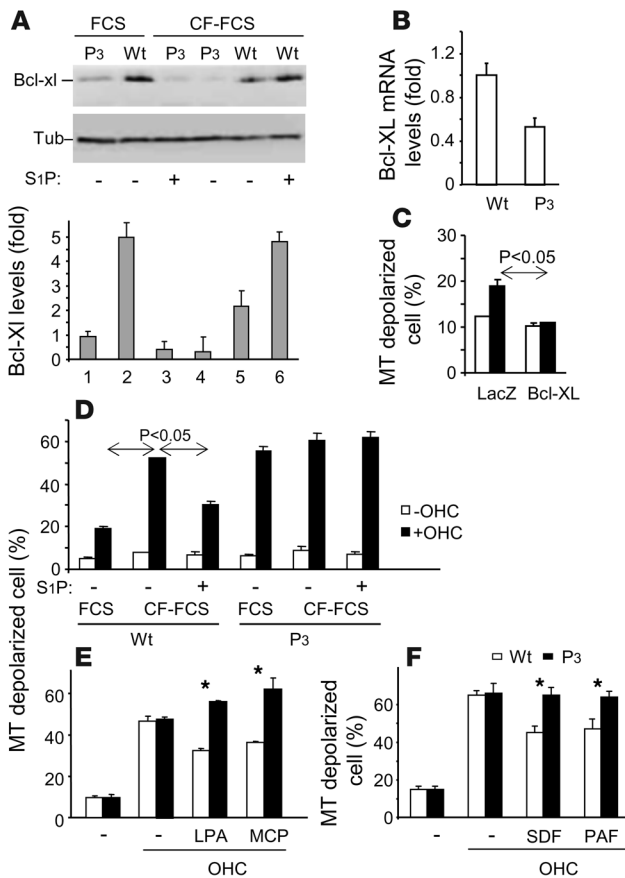


Figure 3

Ligand-induced PLC β 3-dependent upregulation of Bcl-XL in macrophage and apoptosis protection. **(A)** One-day thioglycolate-elicited peritoneal macrophages were cultured in 10% FCS or charcoal-filtered FCS (CF-FCS) in the presence or absence of 300 nM S1P for 24 hours. Cells were then collected for Western blot analyses. Western blots from 3 experiments were quantified by densitometry. **(B)** Relative mRNA levels of Bcl-XL in 1-day thioglycolate-elicited peritoneal macrophages were determined by quantitative RT-PCR. **(C)** One-day thioglycolate-elicited PLC β 3-null peritoneal macrophages were transfected with Bcl-XL expression plasmid using the Amaxa electroporation system. The next day, cells were treated with 25-OHC (2.5 μ g/ml) for 24 hours and then stained with Mito-Probe. **(D)** Cells prepared and treated as in **A** were examined for their sensitivity to 25-OHC (10 μ g/ml). **(E and F)** Cells prepared as in **A** were examined for their sensitivity to 25-OHC in the presence of 10% FCS or charcoal-filtered FCS containing SDF-1 (100 ng/ml), PAF (50 ng/ml), LPA (10 μ M), or MCP (200 ng/ml). * $P < 0.05$ with versus without a ligand.

Bok, Bim, Bik, Bmf, Bax, Bak, Puma, and Bcl-XL in the wild-type and PLC β 3-null macrophages by using specific antibodies. We also examined the levels of p-Mapk, p-Akt, and p-65 NF- κ B, which are also known to play important roles in cell survival (41–43). We did not observe any difference in the levels of these proteins in wild-type and PLC β 3-null macrophages (data not shown) except for Bcl-XL. The level of Bcl-XL in wild-type macrophages was markedly higher than that in PLC β 3-null cells (Figure 3A, compare lanes 1 and 2). Quantitative RT-PCR analysis revealed that this difference may be attributed at least in part to a higher Bcl-XL mRNA level in wild-type than PLC β 3-null macrophages (Figure 3B). Bcl-XL belongs to the antiapoptotic BH domain-containing family of proteins, which also includes Bcl-2 and Mcl-1 (38). The reduction in Bcl-XL expression level in macrophages lacking PLC β 3 would explain the hypersensitivity of these cells to apoptosis induction. The fact that exogenous expression of Bcl-XL in PLC β 3-null peritoneal macrophages attenuated 25-OHC-induced apoptosis supports the explanation (Figure 3C).

As described earlier, a number of ligands are able to regulate PLC β 3 in macrophages. These ligands are abundant in the serum and/or inflammatory tissues (44, 45). It is reasonable to hypothesize that these ligands may regulate Bcl-XL expression in macrophages via PLC β 3. This hypothesis is supported by the finding that when the macrophages were cultured overnight in the presence of serum stripped by charcoal for removal of lipophilic ligands, such as S1P and LPA, the levels of Bcl-XL were markedly reduced compared with those in cells cultured in normal serum (Figure 3A, compare lanes 2 and 5). Importantly, when S1P was added back, it raised the Bcl-XL levels in wild-type macrophages back to those in cells cultured with normal serum, while failing to do so in cells lacking PLC β 3 (Figure 3A). Consistent with what has been shown for the Bcl-XL levels, wild-type macrophages cultured in charcoal-stripped serum showed elevated sensitivity to 25-OHC-induced apoptosis, while PLC β 3-null macrophages showed no significant difference (Figure 3D). Importantly, S1P treatment resulted in a significant reduction in 25-OHC-induced apoptosis of wild-type cells, but not PLC β 3-null cells (Figure 3D). We also examined the effects of SDF-1, MCP-1, PAF, and LPA on macrophage sensitivity to 25-OHC-induced apoptosis. All of these ligands were able to protect wild-type macrophages, but not PLC β 3-null macrophages (Figure 3, E and F), from 25-OHC-induced apoptosis to varying degrees. These ligands also

RAW264.7 cells, in which PLC β 3 expression was suppressed by PLC β 3-specific siRNA (Supplemental Figure 2, E and F), suggesting that this hypersensitivity is not a developmental effect of PLC β 3 gene disruption. We also tested oxLDL, which was prepared following a previously described procedure (32). While our oxLDL preparation showed no effect on wild-type macrophages, it significantly enhanced the apoptosis of PLC β 3-null macrophages (Figure 2F). While some investigators have reported that oxLDL promoted human and mouse macrophage apoptosis (30, 33), others have reported an opposite effect of oxLDL (34, 35). This discrepancy is likely due to different preparations of oxLDL, as lightly oxidized LDL may contain more antiapoptotic than proapoptotic components (36). Our oxLDL preparation may contain equal levels of anti- and proapoptotic components for wild-type cells, but the PLC β 3-null cells may be more sensitive to the proapoptotic components, presumably including oxysterols. The combination of LPS or TNF- α with cycloheximide (CHX) has also been shown to stimulate macrophage apoptosis (37, 38). While LPS/CHX induced greater apoptosis of PLC β 3-null macrophages than wild-type macrophages (Figure 2G), TNF- α /CHX did not (Figure 2H). All of these data together indicate that PLC β 3 signaling may play a significant role in regulating macrophage sensitivity to apoptosis induction by some apoptosis inducers.

Because PLC β 3 deficiency appears to affect mitochondrial potentials measured by the Mito-Probe dye, we hypothesize that PLC β 3 signaling may regulate macrophage apoptosis via regulation of the levels and modifications of BH domain-containing proteins (39, 40). We compared the levels of p-Bad, Bad, Mcl-1, Bcl-2,

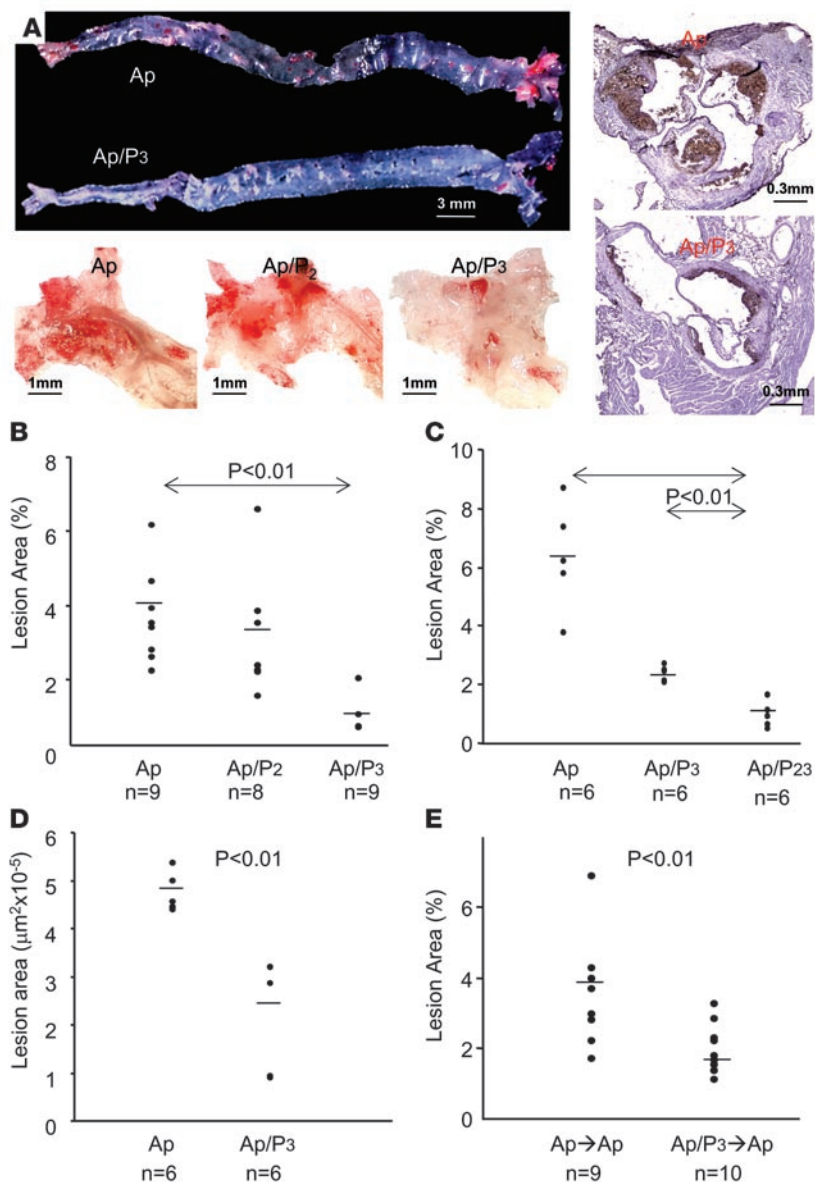


Figure 4 Decreased atherosclerosis in mice lacking PLC β 3. (A) Representative images of en face oil red O staining of spread aortas (top left panel) and aortic arches (bottom 3 left panels) and of Moma-2–stained cross sections of aortic roots (2 right panels) from apoE-null (Ap) and PLC β 3/apoE-null (Ap/P β 3) mice fed an HFD for 10 weeks. (B–E) Quantification of atherosclerotic lesion. apoE-null, PLC β 2/apoE-null, and PLC β 3/apoE-null mice fed an HFD for 10 weeks (B); apoE-null, PLC β 3/apoE-null, and PLC β 2/ β 3/apoE-null (Ap/P β 23) mice fed a high-cholesterol diet for 17 weeks (C and D); and apoE-null mice receiving bone marrows from apoE-null or PLC β 3/apoE-null mice fed an HFD for 14 weeks (E) were analyzed for lesion areas by en face staining of aortas (B, C, and E) or staining of cross sections of aortic roots with oil red O (D).

upregulated the levels of Bcl-XL (data not shown). Thus, PLC signaling is most likely to mediate multiple ligands in protection of macrophages from apoptosis induction in vivo.

Knowing that PLC β 3-deficient macrophages are hypersensitive to apoptosis induction, particularly by 25-OHC and oxLDL, it is reasonable to hypothesize that PLC β 3 deficiency would significantly impact the formation of atherosclerotic lesions, where

oxLDL and oxysterols are enriched. Therefore, we decided to test PLC β deficiency in a mouse atherosclerosis model. Although it is anticipated that the lack of PLC β 3 may increase macrophage apoptosis in the lesions, it is difficult to predict the outcome on atherogenesis itself because of the conflicting literature on the subject. There are reports showing a positive correlation between macrophage apoptosis and atherogenesis and ones showing the opposite (46–50). We favored the prediction that PLC β 3 deficiency may be associated with a reduction in atherogenesis based on the finding that inactivation of Bax, which Bcl-XL counteracts, attenuated macrophage apoptosis and accelerated atherogenesis in a mouse model (48). Thus, we hoped that this atherosclerosis model study would not only provide a critical in vivo validation of what we have observed in isolated primary macrophages but also help to further clarify the role of macrophage apoptosis in atherogenesis. To investigate the effect of PLC β 3 deficiency on atherogenesis, the PLC β 3-null mice were first backcrossed to the C57BL background and then crossed with apoE-null mice, a well-established mouse atherosclerosis model (51, 52). The PLC β 3/apoE-double-null mice, together with the apoE-null mice, were fed an HFD for 10 or 17 weeks. As shown in Figure 4A, PLC β 3 deficiency led to marked reductions in atherosclerotic lesions in aortic vessels, arches, and roots compared with those in apoE-null mice. Quantification of lesion areas detected by en face oil red O staining of aortas revealed that there was a roughly 70% reduction in the lesion areas (Figure 4B). In addition, there were marked reductions in Moma-2 (a foam cell marker) staining at the aortic roots (Figure 4A). Serum lipid profiles of PLC β 3/apoE-null mice were also compared with those of apoE-null mice; no significant differences in the levels of cholesterol, LDL, or triglyceride (Supplemental Figure 3A) were observed. A significant reduction (by 70%) in atherosclerotic lesion was also observed in PLC β 3-null mice fed an HFD for 17 weeks when compared with apoE-null mice (Figure 4C). Lesions in aortic roots of these mice were also quantified; consistent with en face quantification, a significant reduction (by more than 50%) in the lesion was observed (Figure 4D). Once again, no significant changes in lipid profiles were found to be caused by PLC deficiency (Supplemental Figure 3B).

We also examined the effects of PLC β 2 deficiency and PLC β 2/ β 3 double deficiency on atherogenesis. PLC β 2/apoE-double-knock-out mice were generated by crossing PLC β 2-null mice, which are also in the C57BL background, with apoE-null mice, and PLC β 2/PLC β 3/apoE-triple-knockout mice were subsequently generated by additional crossing. Although PLC β 2 deficiency did not have a significant effect by itself (Figure 4, A and B), PLC β 2/ β 3

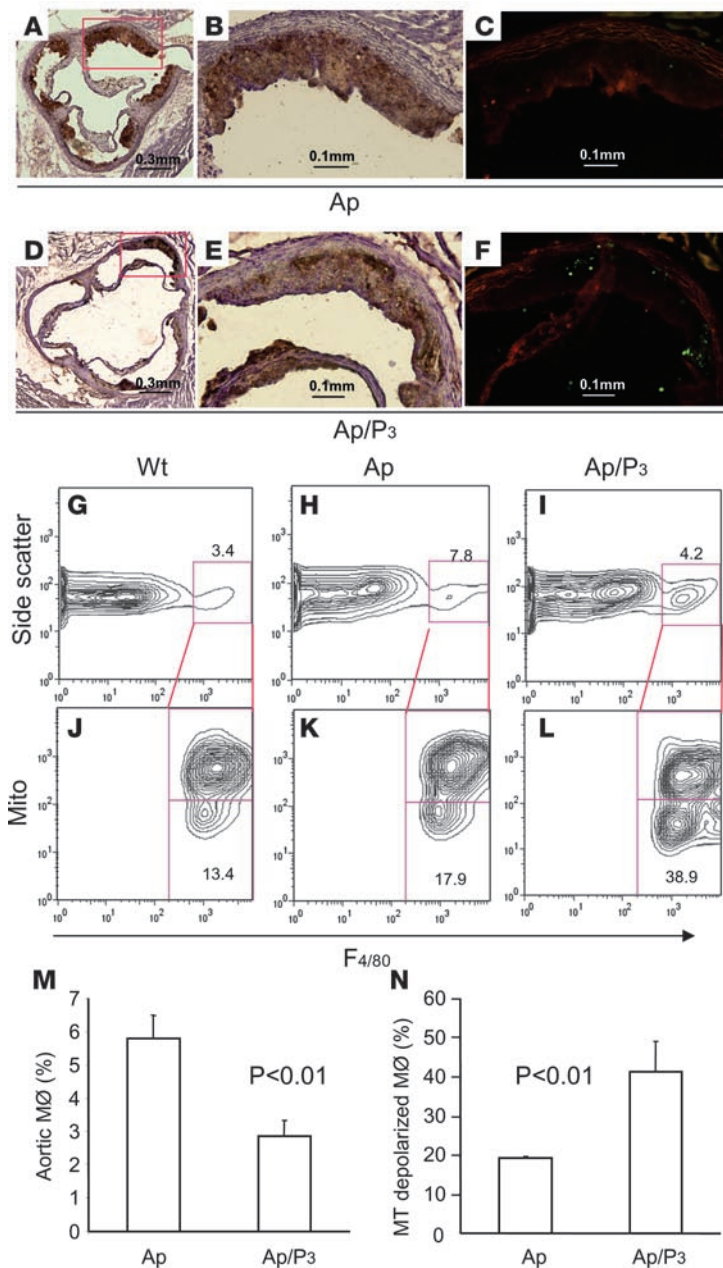


Figure 5 PLC $\beta 3$ deficiency is associated with increased macrophage apoptosis in the atheromas. (A–F) Representative images of the cross sections of aortic roots from mice fed an HFD for 10 weeks stained with Moma-2 antibody (A, B, D, and E) or by TUNEL (C and F). B and E show magnifications of squared regions in A and D. C and F show adjacent sections of B and E, respectively. (G–N) Aortas from mice on an HFD for 17 weeks (G–L) or 10 weeks ($n = 7$; M and N) were analyzed for the number of macrophages and apoptotic macrophages.

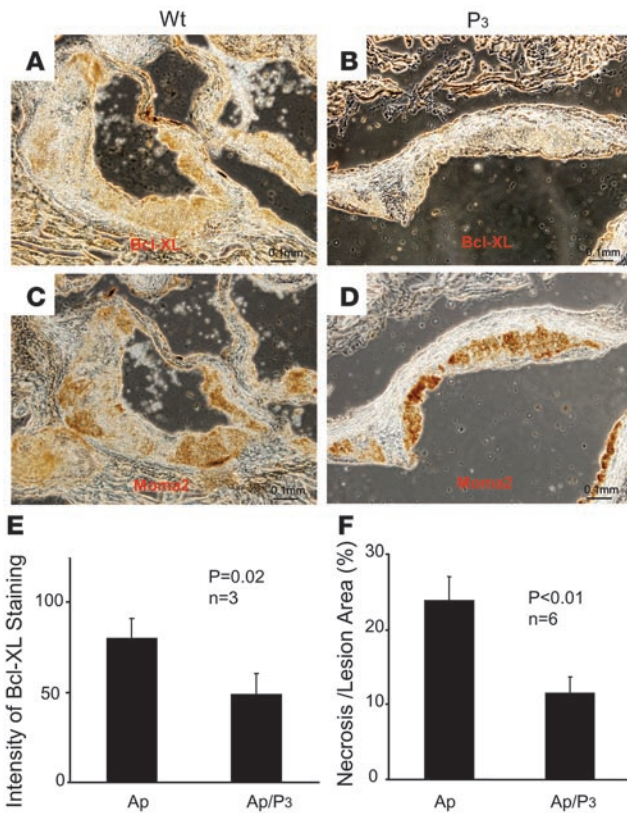
double deficiencies resulted in a greater reduction in atherosclerotic lesion formation than PLC $\beta 3$ deficiency did (Figure 4C). PLC $\beta 3$ is widely expressed, but PLC $\beta 2$ expression is relatively more restricted to hematopoietic cells. This synergistic effect of PLC $\beta 2$ deficiency suggests that the lack of PLC in hematopoietic cells may contribute to the reduction in atherogenesis. In addition, these effects of PLC deficiencies on atherogenesis are consis-

tent with their effects on ligand-induced Ca^{2+} effluxes in macrophages shown in Figure 1A, i.e., PLC $\beta 3$ deficiency showed a greater effect than PLC $\beta 2$ deficiency did, while PLC $\beta 2/\beta 3$ double deficiencies had the strongest effect. This correlation between the atherogenic phenotypes and Ca^{2+} efflux responses suggests that the effects of PLC deficiencies on atherogenesis may be attributed to their effects on macrophage functions.

To provide additional evidence for the lack of PLC $\beta 3$ in hematopoietic cells being responsible for the atherogenic phenotypes of PLC $\beta 3$ deficiency, we carried out adoptive bone marrow transfer experiments. Bone marrow cells from apoE-null and PLC $\beta 3$ /apoE-null mice were transferred to recipient apoE-null mice that had been lethally irradiated. As shown in Figure 4E, in mice that received PLC $\beta 3$ -null bone marrow cells, aortic lesions areas were more than 50% smaller than in mice that received cells expressing PLC $\beta 3$. A similar result was seen in aortic root lesions (data not shown), and no difference in lipid profiles were noted (Supplemental Figure 3C). The bone marrow transplantation efficiency was verified by Western blot analysis of bone marrow cells using an anti-PLC $\beta 3$ antibody; the PLC $\beta 3$ protein was hardly detected in bone marrow cells isolated from mice that received PLC $\beta 3$ -null bone marrow (Supplemental Figure 3D). We also performed the transfer of apoE-null bone marrow cells to lethally irradiated apoE- and apoE/PLC $\beta 3$ -null recipients, respectively; no significant differences in their lesion areas were observed (Supplemental Figure 3E). All these results, together with the lack of effects of PLC $\beta 3$ deficiency on chemokine-induced Ca^{2+} in spleen T and B cells (data not shown), indicate that PLC deficiency in hematopoietic cells, most likely macrophages, leads to a reduction in atherogenesis, which is in agreement with our hypothesis.

The next key question was whether PLC $\beta 3$ deficiency increases macrophage apoptosis in atherosclerotic lesions. We first evaluated apoptosis in atherosclerotic lesions by staining adjacent sections of aortic roots from apoE-null and apoE/PLC $\beta 3$ -double-null mice with TUNEL and the anti-Moma-2 antibody, respectively (Figure 5, A–F, and Supplemental Figure 4). We found that there was more TUNEL-positive staining in the section from apoE/PLC $\beta 3$ -null mice than that from apoE-null mice (Figure 5, C and F), even though the lesion size of the apoE/PLC $\beta 3$ sample was smaller (Figure 5, B and E). To carry out a more reliable and objective quantification of the number of macrophages and apoptotic macrophages in the lesions, we developed a FACS-based assay modified from a method reported by Ley's group (53). Cells dispersed from aortas of wild-type, apoE-null, and apoE/PLC $\beta 3$ -null mice (apoE- and apoE/PLC $\beta 3$ -null mice were on an HFD for 17 weeks) after collagenase A digestion were stained with

the Mito-Probe dye and an anti-F4/80 antibody and subjected to FACS analysis. When compared with the wild-type samples, there was a marked increase in the number of macrophage (F4/80-positive) cells in the apoE-null samples (Figure 5, G and H). This is consistent with the current knowledge of increased infiltration of macrophages in atherosclerotic lesions. Importantly, the number of macrophages in the apoE/PLC $\beta 3$ -null sample was less than

**Figure 6**

Effect of PLC $\beta 3$ deficiency on Bcl-XL expression in the lesions and on necrotic core size. Adjacent aortic root sections of apoE- (A and C) and apoE/PLC $\beta 3$ -null (B and D) mice fed an HFD were stained with an anti-Bcl-XL antibody in parallel (A and B) or the anti-Moma-2 antibody (C and D). (E) Quantification of Bcl-XL staining ($n = 6$). (F) The necrotic core size in aortic root sections from mice on an HFD for 17 weeks was quantified.

increase in apoptotic macrophages in atherosclerotic lesions, probably as the result of reduction in Bcl-XL levels, which in turn contributes to the reduction in the number of macrophages in the lesions as well as the lesion size.

Discussion

Our work has demonstrated that PLC $\beta 3$, a major functional PLC isoform that is activated by a number of G protein-coupled ligands in macrophages, plays an important role in regulating macrophage sensitivity to apoptotic induction by oxysterols and LPS. PLC signaling regulates the sensitivity at least in part by regulating the expression of Bcl-XL. We subsequently validated the *in vivo* significance of this PLC $\beta 3$ -mediated mechanism in a mouse atherosclerosis model. In this model study, we found that PLC $\beta 3$ deficiency was associated with significant reductions in atherogenesis and increases in macrophage apoptosis in atherosclerotic lesions and sensitivity to apoptotic induction *in vitro*.

It is well accepted that chemokines and their receptors have important roles in atherogenesis (9, 11–13), and these roles of chemokines have been largely attributed to their regulation of macrophage recruitment. The finding that SDF-1 and, to a lesser extent, MCP-1 can protect macrophages from apoptosis induction, however, suggests that chemokines may regulate atherogenesis via diverse mechanisms. While S1P and LPA can activate PLC via both the Gq and Gi family of proteins, chemokines including both MCP-1 and SDF-1 activate PLC primarily via the Gi proteins in leukocytes (54–56). PLC $\beta 3$ was shown to be activated by both the $G\alpha$ subunits of the Gq family and $G\beta\gamma$ subunits. Thus, it is reasonable to observe that all these ligands are able to protect macrophages from apoptotic induction in a PLC $\beta 3$ -dependent manner. It also appears that PKC has an important role in PLC $\beta 3$ -mediated regulation of macrophage apoptosis, as a PKC inhibitor, Go6976, inhibited serum-mediated protection of macrophage apoptosis induced by 25-OHC (data not shown).

It is also important to note that PLC $\beta 3$ deficiency did not by itself cause excessive cell death of either macrophages (Figure 1E) or monocytes (data not shown), nor did it alter the numbers or apoptotic levels of monocytes in the spleen, bone marrow, and circulation even in the apoE-null background. These observations suggest that this PLC signaling-mediated antiapoptotic mechanism may either be specific for macrophages or function under a specific context where PLC-activating ligands and proapoptotic stimuli are enriched. Thus, it is reasonable to find that this mechanism is highly relevant in the atherosclerotic lesions, in which both apoptotic inducers (oxysterols) and PLC-activating ligands (chemokines, S1P, LPA, and PAF) are abundantly present. It is conceivable to postulate that macrophages develop this protective mechanism to cope with harsh environments these cells often encounter as the primary scavenger cells. Such harsh environments are presumably found in inflammation and microbial infections. Although this protective mechanism, particularly the resistance

that in the apoE-null sample (Figure 5, H and I), but slightly more than that in the wild-type mouse (Figure 5, G and I). These results agree well with significant reduction in atherosclerotic lesions in the apoE/PLC $\beta 3$ -null mice (Figure 4). Even though the apoE/PLC $\beta 3$ -null sample had fewer macrophages than the apoE-null one, it had an increased percentage of F4/80-positive macrophages that were mitochondria depolarized compared to the one lacking only apoE (Figure 5, K and L). On the other hand, the proportion of apoptotic cells in the apoE-null aorta was just slightly higher than that in the wild-type aorta (Figure 5, compare K and J). These data suggest that there is a marked increase in the apoptosis of PLC $\beta 3$ -null macrophages in the aortic lesions, which is correlated with a reduction in atherogenesis. We analyzed another set of wild-type, PLC $\beta 3$ /apoE-null, and apoE-null samples from mice on an HFD for 17 weeks, and the results were the same (data not shown). To obtain statistical analysis, we carried out a similar experiment using 7 pairs of apoE- and apoE/PLC $\beta 3$ -null mice fed an HFD for 10 weeks. The results, which are shown in Figure 5, M and N, were the essentially same. To examine whether there are differences in the Bcl-XL expression levels between the wild-type and PLC $\beta 3$ -null lesion areas, we stained aortic root sections with an anti-Bcl-XL antibody. Significant differences in Bcl-XL staining between the wild-type and PLC $\beta 3$ -null sections were observed (Figure 6, A–E). We also stained the adjacent sections with the anti-Moma-2 antibody. Bcl-XL was readily detected in the macrophages in the lesions from the wild-type mice. We also quantified the necrosis in the lesions of mice fed an HFD for 17 weeks. There was less necrosis in lesions from PLC $\beta 3$ -null mice than those from the wild-type mice (Figure 6F). Therefore, we conclude that PLC $\beta 3$ deficiency is associated with a significant



to LPS-induced apoptosis (Figure 2G), may be beneficial in anti-microbial actions and even acute inflammation, the mechanism appears to be counterproductive during atherogenesis and maybe other chronic inflammatory conditions. Thus, it is of great interests to investigate the significance of PLC $\beta 3$ signaling in other inflammation-related paradigms.

The roles of macrophage apoptosis in atherogenesis have been investigated in a number of recent studies. It seems that macrophage apoptosis may show different effects on atherogenesis depending on the stages at which it occurs. An increase in macrophage apoptosis in early lesions appears to cause the attenuation of atherogenesis, whereas impairment in macrophage apoptosis in late lesions may contribute to necrosis, leading to increased proinflammatory responses and atherogenesis (47–50, 57, 58). Given that there is no increase in necrotic core size associated with PLC $\beta 3$ deficiency, our results are consistent with the idea that the lack of PLC $\beta 3$ enhances the sensitivity of newly recruited macrophages to 25-OHC- or oxLDL-induced apoptosis in early atherosclerotic lesions, which causes them to decrease before they become foam cells, the building blocks for atherosclerotic plaques. Fewer foam cells would mean smaller lesions. However, because of the early lesion suppression by PLC $\beta 3$ deficiency, it is difficult to draw any conclusion on the direct effect of the PLC signaling-regulated macrophage apoptosis on advanced lesion progression. This issue may have to be addressed by temporal or pharmacological control of PLC $\beta 3$ expression in future. Although we have not found any effect of PLC $\beta 3$ deficiency on the macrophage activities other than apoptosis, our present study also cannot completely exclude the possibility that some unknown macrophage functions mediated by this PLC pathway contribute to the atherogenic phenotypes. Nevertheless, our study has established a clear correlation between the absence of PLC $\beta 3$ on the one hand and an increase in macrophage apoptosis and reduction in atherogenesis on the other. Although we have only studied mouse macrophages, recent work showing that S1P induced upregulation of Bcl-XL and Bcl-2 in human macrophages and protected human macrophages from apoptosis induction (38) provides the possible relevance for our study for humans. Together with the fact that PLC $\beta 3$ deficiency is associated with no apparent gross phenotypes, we believe that PLC $\beta 3$ may be a potential antiatherosclerosis target.

Methods

Animals. PLC $\beta 2$ -null and PLC $\beta 3$ -null mice were backcrossed with C57BL/6J mice for more than 10 generations. These mice were then crossed with each other to produce PLC $\beta 2/\beta 3$ -null mice and with apoE-null mice in the C57BL/6J background purchased from the Jackson Laboratory (Apoetm1Unc/J). For analysis of atherogenesis, mice were fed an HFD (Harlan Teklad) starting at 5 weeks of age for 10 or 17 weeks. All animal procedures used in this study followed institutional guidelines of the University of Connecticut Health Center and Yale University and were approved by the University of Connecticut Health Center Animal Care Committee and the Yale University Institutional Animal Care and Use Committee.

Cells, plasmids, chemoattractants, and antibodies. Raw264.7 cells were cultured in DMEM (Cellgro) supplemented with 10% FCS (Hyclone; heat-inactivated), penicillin and streptomycin. Raw264.7 cells were transfected using Lipofectamine (Invitrogen). Expression plasmids for LacZ, PAK1, and the kinase-dead mutant of PAK1 were described previously (59).

Peritoneal macrophages were prepared 24 hours or 72 hours after mice were injected with 1 ml of 3% thioglycolate broth (Sigma-Aldrich). Peri-

toneal cavities were lavaged with 10 ml HBSS (Gibco; Invitrogen), and an aliquot of cells was stained with the anti-F4/80 antibody. Generally, 90% of the cells isolated after 72 hours were F4/80 positive, and more than 60% were F4/80 positive after 24-hour induction.

SDF-1 and MCP-1 were purchased from PeproTech Inc. C5a was purchased from Sigma-Aldrich. Anti-Bcl-XL antibody and anti-PAK1 antibodies were purchased from Cell Signaling Technology Inc. Anti-phospho-JNK antibody was purchased from United Biomedical Inc. Anti-Moma-2 antibody was purchased from Serotec Inc. CD11b and Gr-1 antibody were purchased from BD Biosciences – Pharmingen. F4/80 antibody was purchased from Serotec Inc.

Calcium efflux assay. Peritoneal macrophages of WT and PLC $\beta 2$ -, PLC $\beta 3$ -, and PLC $\beta 2/\beta 3$ -null mice were collected and labeled with Fluo-3. Cell suspensions were collected in a flow cytometer for 30 seconds before the C5a was added into suspension. Calcium intensity was recorded in real time.

Macrophage apoptosis assays. To analyze macrophage apoptosis, cells were stained with annexin V and propidium iodide using the ApoAlert AnnexinV-FITC Apoptosis Kit (Clontech) or with Mito-Probe DiIC1 dye (Invitrogen), and the anti-F4/80 antibody and analyzed by FACS. The caspase activity assays were performed using a Homogeneous Caspase Assay from Roche based on the manufacturer's suggested protocol.

To test macrophages' sensitivity to 25-OHC or oxLDL, cells were incubated with or without 10 $\mu\text{g}/\text{ml}$ 25-hydroxycholesterol (Sigma-Aldrich) or 100 $\mu\text{g}/\text{ml}$ oxLDL in DMEM containing 10% FCS for 24 hours at 37°C and analyzed for apoptosis. For examination of the effects of S1P, cells were resuspended in DMEM containing 10% charcoal-filtered FCS (Gemini Bio-Products) and treated with or without 300 nM S1P in the presence or absence of 10 $\mu\text{g}/\text{ml}$ 25-OHC. Cells were analyzed for apoptosis 24 hours later.

For analysis of Raw264.7 cells, the cells that were transfected for 24 hours were incubated with 30 $\mu\text{g}/\text{ml}$ 25-OHC for an additional 24 hours before they were analyzed by FACS with the Mito-Probe dye.

Quantification of macrophage number and apoptosis in aortas. Aortas were quickly dissected as described in *Atherosclerotic lesion analysis*, and were minced and incubated with 1 mg/ml collagenase A (Roche) for 2 hours at 37°C. Then, the cells were gently spun down and stained with the anti-F4/80 antibody and Mito-Probe dye and analyzed by FACS.

Sample preparation for Western blot analysis. Peritoneal macrophages were cultured in DMEM containing 10% charcoal-filtered or normal FCS in the presence or absence of 10 $\mu\text{g}/\text{ml}$ 25-OHC or 300 nM S1P for 24 hours at 37°C before being directly lysed with the SDS-PAGE sample buffer.

Cell migration assays. Cell migration assays were performed using 24-well Transwell plates (5- μm pore size; Costar). Two million peritoneal macrophages or 5 million spleen cells were loaded into the upper chambers. The lower chambers were filled with medium (DMEM plus 1% FCS) in the absence or presence of chemoattractants. In some experiments, Transwell inserts were cultured with a layer of mouse embryonic immortalized endothelial cells (60). Transwell plates were then incubated at 37°C for 4 hours. For peritoneal macrophage migration assays, cells attached to the lower surfaces of inserts were trypsinized and collected for counting and FACS analysis after staining with the anti-F4/80 antibody. For quantification of spleen monocyte migration, cells in the lower chambers were collected for counting and FACS analysis after being stained with anti-CD11b and anti-Gr-1 antibodies. The number of monocytes was determined based on the total migrated cell number and percentage of monocytes (CD11b^{mid} and Gr-1^{lo}CD11b^{mid}).

Adhesion assay. Five million spleen cells were added into tissue culture plates (Costar) without coating or precoated with 50 $\mu\text{g}/\text{ml}$ poly-lysine, 1 $\mu\text{g}/\text{ml}$ fibronectin (Sigma-Aldrich), or a layer of mouse endothelial



cells. The plates were then incubated in 37°C for 10 minutes. After cells were washed with PBS 5 times, adherent cells were detached by trypsinization, counted, and analyzed by FACS after staining with anti-CD11b and anti-Gr-1 antibodies.

Phagocytosis assay. Peritoneal macrophages were seeded to culture plates for 3 hours at 37°C. Uncoated FluoSphere beads (Molecular Probes; Invitrogen) and those coated with oxLDL were added to the cultures after unattached cells were rinsed away. After 10 minutes of incubation at 37°C, excessive beads were washed away, and the macrophages were detached by trypsinization and analyzed by FACS after being stained with the anti-F4/80 antibody.

For the phagocytosis assay of apoptotic Jurkat cells, the apoptosis was induced by incubating Jurkat cells with an anti-Fas antibody (Upstate) for 6 hours in 37°C. Apoptosis was verified by annexin V/propidium iodide staining. Apoptotic Jurkat cells were then labeled with Vybrant CFDA SE (Molecular Probes; Invitrogen) and added to peritoneal macrophages. After 3 hours of incubation at 37°C, the unbound Jurkat cells were washed away before macrophages were detached and stained with F4/80. The cells were analyzed by a flow cytometer. The mean fluorescence intensity of CFDA SE gated for F4/80-positive cells is shown as the number of apoptotic Jurkat cells associated with macrophages.

Atherosclerotic lesion analysis. Mice were anesthetized, and blood samples were collected via heart puncture for lipid profile analyses (total cholesterol, LDL, HDL, and triglycerides) by IDEXX Veterinarian Laboratories). Then the cardiovascular system was perfused with 20 ml 1× DPBS (Cellgro), through the heart apex, followed by 5 ml fixatives (4% PFA, 5% sucrose, 20 μM EDTA in 1× DPBS, pH 7.4). The aorta from the aortic root to iliac artery was dissected and cut open longitudinally in situ and immersed in the fixative for 12 hours before being rinsed with 1× PBS and stained with oil red O (Fisher Biotech). The aorta was then spread, and the lesion area was quantified using the imaging analysis software ImagePro from Media Cybernetics. The heart and proximal portion of aortic root were also fixed and embedded in OCT for lesion area quantification on cross sections of the aortic root, as previously described (61).

Bone marrow transplantation. Recipient apoE-null mice were lethally irradiated with 5.50 Gy twice at a 4-hour interval. Twenty four hours later, the mice were transplanted via tail vein injection with bone marrow cells (2 million/recipient) from apoE-null mice or apoE/PLC β3-null mice. The bone marrow cells were prepared by flushing femurs with 1× DPBS and lysing red blood cells with rbc lysis buffer (8.3 g NH₄Cl, 1.0 g KHCO₃, 1.8 ml 5% EDTA in 1,000 ml dH₂O). Four weeks after bone marrow transfer, recipient mice were fed an HFD for 10 weeks for atherosclerotic lesion analysis. Before mice were euthanized, bone marrow cells were collected for Western blot detection of PLC β3 protein to monitor transplantation efficiency.

Immunohistochemistry and TUNEL staining. For Moma-2 and Bcl-XL staining, frozen sections were fixed with acetone/methanol (vol/vol 1:1) for 30 minutes before being transferred to 1× TBS (8.766 g/l NaCl, 6.055 g/l Tris, pH 7.4) for 10 minutes at room temperature. Sections were then immersed in 0.3% H₂O₂, rinsed, and transferred into 10% normal rabbit serum (Vector Laboratories), followed by staining with rat anti-mouse Moma-2 antibody, biotinylated second antibody, and HRP-avidin conjugates using the ABC kit from Vector Laboratories. Finally, the sections were developed with DAB (Dako North American), and some of the sections were counterstained with hematoxylin (Thermo Electron Corp.). Bcl-XL staining intensity was quantified by using MetaMorph software in circumscribed areas of atherosclerotic lesions.

For TUNEL staining, the sections were stained with the TACS In Situ Kit (R&D Systems) based on the manufacturer's suggestions. Briefly, sections were immersed in PBS for 15 minutes at room temperature,

incubated with protease K for 15 minutes at room temperature, transferred into the TdT labeling buffer, and incubated with the TdT labeling reaction mix. Labeling was stopped by transferring sections to the stop buffer, and the sections were incubated in Strep-Fluor. All reagents were provided in the kit.

Expression of Bcl-XL in primary macrophages. Macrophages isolated from mouse peritonea were cotransfected with the EGFP and Bcl-XL expression plasmids at a ratio of 1:2 using the Amaxa electroporation system. Twenty-four hours after transfection, cells were collected and treated with 25-OHC, stained with the Mito-Probe dye, and analyzed by a flow cytometer as described above. The GFP-positive cell population was gated in the analysis of apoptosis. The transfection efficiency was around 10%. The control group was primary macrophages transfected with EGFP plus LacZ.

Quantitative RT-PCR. Total RNA was isolated from macrophages of WT and PLC β3-null mice using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For quantitative PCR analysis, RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was carried out using the QuantiTect SYBR Green PCR kit (QIAGEN) on a DNA Engine OPTICON (MJ Research Inc.) instrument. β-Actin was used as an internal reference for each sample. Using a formula previously described (62), the relative change in mRNA levels was normalized against the β-actin mRNA levels. The sense primer was 5'-CTGTGC-GTGGAAAGCGTAGA-3', and the antisense primer was 5'-GTCAGAAC-CAGCGTTGAA-3'.

PLC β3 siRNA expression in RAW264.7 cells. RAW264.7 cells were transfected with either pAS control vector or pAS-PLC β3 by using Lipofectamine reagent (Invitrogen). Forty-eight hours after transfection, the cells were collected and treated with 25-OHC and analyzed for apoptosis as described above. During analysis, only the GFP-positive cell population was gated in the analysis of apoptosis.

Quantification of necrotic core size. A modification of a previously described method (49) was performed to measure necrosis in atherosclerotic lesions. The frozen tissue blocks were placed on a cryotome, and 6-μm serial sections of the ascending aorta were collected on coated glass slides until the most cranial portion of the aortic sinus could be identified by examining unstained sections. Once the first section was identified, the 36 cranial sections, covering 216 μm of the ascending aorta, were used for further evaluation. Every twelfth section of the ascending aorta was stained with H&E. The necrosis areas were circumscribed by using MetaMorph software (Molecular Devices) based on characteristic morphologic features of necrosis. The area of necrosis of each slide was calculated and normalized to the total lesion area.

Statistics. For atherosclerotic lesion area analysis, both 2-tailed Student's *t* test and Mann-Whitney *U* test were performed. The *P* values generated by both analyses were consistent. For the rest of the assays, Student's *t* test was used to calculate the statistical significance. *P* < 0.05 was considered significant.

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