Cytosolic phospholipase $A_2\alpha$–deficient mice are resistant to experimental autoimmune encephalomyelitis

Suzana Marusic,[1] Michael W. Leach,[2] Jeffrey W. Pelker,[1] Mihai L. Azoitei,[1] Naonori Uozumi,[3] Junqing Cui,[1] Marina W.H. Shen,[1] Charlene M. DeClercq,[1] Joy S. Miyashiro,[1] Brenda A. Carito,[2] Paresh Thakker,[1] David L. Simmons,[1] John P. Leonard,[1] Takao Shimizu,[3] and James D. Clark[1]

[1]Department of Inflammation, Wyeth Research, Cambridge, MA 02140
[2]Exploratory Drug Safety, Wyeth Research, Andover, MA 01810
[3]Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Tokyo, Tokyo 113-0033, Japan

Experimental autoimmune encephalomyelitis (EAE), a Th1-mediated inflammatory disease of the central nervous system (CNS), is a model of human multiple sclerosis. Cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$), which initiates production of prostaglandins, leukotrienes, and platelet-activating factor, is present in EAE lesions. Using myelin oligodendrocyte glycoprotein (MOG) immunization, as well as an adoptive transfer model, we showed that $cPLA_2\alpha^{-/-}$ mice are resistant to EAE. Histologic examination of the CNS from MOG-immunized mice revealed extensive inflammatory lesions in the $cPLA_2\alpha^{+/-}$ mice, whereas the lesions in $cPLA_2\alpha^{-/-}$ mice were reduced greatly or completely absent. MOG-specific T cells generated from WT mice induced less severe EAE in $cPLA_2\alpha^{-/-}$ mice compared with $cPLA_2\alpha^{+/-}$ mice, which indicates that $cPLA_2\alpha$ plays a role in the effector phase of EAE. Additionally, MOG-specific T cells from $cPLA_2\alpha^{-/-}$ mice, transferred into WT mice, induced EAE with delayed onset and lower severity compared with EAE that was induced by control cells; this indicates that $cPLA_2\alpha$ also plays a role in the induction phase of EAE. MOG-specific T cells from $cPLA_2\alpha^{-/-}$ mice were deficient in production of Th1-type cytokines. Consistent with this deficiency, in vivo administration of IL-12 rendered $cPLA_2\alpha^{-/-}$ mice susceptible to EAE. Our data indicate that $cPLA_2\alpha$ plays an important role in EAE development and facilitates differentiation of T cells toward the Th1 phenotype.

CORRESPONDENCE
Suzana Marusic:
smarusic@wyeth.com

Abbreviations used: 5-LO, 5-lipoxygenase; AA, arachidonic acid; AACOCF3, arachidonyl trifluoromethylketone; CNS, central nervous system; $cPLA_2\alpha$, cytosolic phospholipase $A_2\alpha$; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; LT, leukotriene; MCP, monocyte chemoattractant protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PAF, platelet-activating factor; PG, prostaglandin.

Experimental autoimmune encephalomyelitis (EAE) is a T cell–mediated inflammatory disease of the central nervous system (CNS), which clinically manifests as ascending paralysis. It can be induced in susceptible animals by immunizing them with myelin proteins or by injecting them with myelin protein–specific CD4$^+$ T cells. EAE shares many clinical and pathologic features with multiple sclerosis (MS), and is a commonly used animal model of this human autoimmune disease (1–3).

MS and EAE are believed to be Th1-induced autoimmune diseases because of the increased expression of Th1 cytokines in the affected CNS and because injection of myelin-specific Th1, but not Th2, cells into immunocompetent mice is sufficient to induce EAE (4–9). Members of the Th1-inducing family of cytokines, IL-12, -23, and -27, seem to be important for Th1 differentiation and EAE development (10–12). IL-12, composed of p35 and p40 subunits, is a prototypic Th1 cytokine that induces differentiation of Th1 cells (13, 14). Its role in EAE was proposed based on studies that showed that adding IL-12 to the cultures of the encephalitogenic cells greatly exacerbated EAE, whereas administration of anti-p40 antibodies ameliorated EAE (10, 15). However, later studies suggested that IL-23, which has the same p40 subunit as IL-12, may be more critical than IL-12 in the development of EAE (11, 16). Recent studies using anti-p28 antibodies, which block IL-27, suggested that IL-27 also may be necessary for EAE development (12).

In addition to Th1-type cytokines, many other mediators of inflammation are increased

J.P. Leonard's present address is Genzyme Drug Discovery & Development, Waltham, MA 02451.
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during EAE and MS development; it is likely that a subset of these may be therapeutically useful targets in MS. Lipid mediators of inflammation, including prostaglandins (PGs), leukotrienes (LTs), and platelet-activating factor (PAF), are elevated in the cerebrospinal fluid (CSF) of patients who have MS (17–19). Cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) initiates the production of these inflammatory mediators (20, 21); is expressed by endothelial cells, $CD4^+$ T cells, and macrophages at the site of EAE lesions (22); and is activated by monocyte chemoattractant protein (MCP)-1 and -3, monocyte inflammatory protein–1α, and regulated on activation, normal T cell expressed and secreted (23, 24), which are chemokines that are believed to play a role in EAE and MS (25). These chemokines stimulate the phosphorylation and translocation of $cPLA_2\alpha$, and lead to selective release of arachidonic acid (AA) from phospholipid membranes. The free AA is converted to PG by way of the cyclooxygenase pathway, and LTB_4 and cysteinyl leukotrienes by way of the 5-lipoxygenase (5-LO) pathway. Concurrent with the release of AA, lysophosphatidylcholine is generated, which serves as the precursor for the proinflammatory PAF (26, 27). In addition to being converted into proinflammatory mediators, AA can be converted into lipoxins that may promote resolution of inflammation (28, 29) or peroxisome proliferator-activated receptor γ agonists, including 15-hydroxyeicosatetraenoic acid (30). Agonists of peroxisome proliferator-activated receptor γ were shown to regulate macrophage and T cell functions negatively and to reduce the severity of EAE (31).

The roles of different end-products of $cPLA_2\alpha$ enzymatic activity in EAE development are not well understood, but Kalyvas and David (22), who documented the presence of $cPLA_2\alpha$ at the site of lesions, also demonstrated that arachidonyl trifluoromethylketone (AACOCF3)—which inactivates $cPLA_2\alpha$ by reversible reaction with the active site serine (32)—can reduce EAE severity in myelin oligodendrocyte glycoprotein (MOG)-immunized mice. AACOCF3 was effective at blocking the onset of disease, and, in some cases, preventing relapse. However, AACOCF3 also inhibits calcium-independent phospholipase A_2 (33), and thromboxane synthase, which is downstream of cyclooxygenase (32). AACOCF3 also inhibits fatty acid amide hydrolase, the enzyme that degrades the natural ligands for the cannabinoid receptors (34, 35), and cannabinoid receptor agonists ameliorate clinical EAE and suppress inflammatory cytokines in a Theiler virus–induced model of EAE (36). Thus, AACOCF3 may be acting on multiple enzymes, and therefore, its effect in EAE may not be due solely to inhibition of $cPLA_2\alpha$.

To assess the role of $cPLA_2\alpha$ in EAE, we established the $cPLA_2\alpha^{-/-}$ mice on the susceptible B6 background, and studied the development of disease in $cPLA_2\alpha^{-/-}$ and $cPLA_2\alpha^{+/-}$ littermates. We found that $cPLA_2\alpha^{-/-}$ mice are resistant to EAE induction. We demonstrated that this resistance is accompanied by a defect in development of Th1 responses and can be restored by administration of IL-12.

RESULTS

$cPLA_2\alpha^{-/-}$ mice do not develop EAE after immunization with MOG

To test if end-products of $cPLA_2\alpha$ enzymatic activity were increased during EAE development, we measured products of the cyclooxygenase and 5-LO pathways in spinal cords of naive mice, as well as mice at the onset, peak, and recovery of the disease. Very low levels of these mediators were found in the spinal cords of naive mice. Conversely, levels of PGE_2 and LTB_4 were increased significantly ($P < 0.05$) at the peak and during the recovery phase of EAE (Fig. 1).

To test the potential role of $cPLA_2\alpha$ in EAE development, we immunized $cPLA_2\alpha^{-/-}$ or $cPLA_2\alpha^{+/-}$ littermates with MOG and followed EAE development. In three independent experiments, $cPLA_2\alpha^{-/-}$ mice were resistant to EAE induction (3 out of 27 mice developed EAE), whereas $cPLA_2\alpha^{+/-}$ mice developed severe EAE (22 out of 23 mice developed EAE) (Fig. 2). There was no difference in the severity of EAE between $cPLA_2\alpha^{+/-}$ and $cPLA_2\alpha^{+/+}$ littermates (unpublished data); therefore, the $cPLA_2\alpha^{+/-}$ littermates were used as controls for $cPLA_2\alpha^{-/-}$ mice in all further experiments. Microscopic examination of the brain and spinal cord showed remarkable differences between the $cPLA_2\alpha^{+/-}$ and $cPLA_2\alpha^{-/-}$ mice (Fig. 3; Table I). The $cPLA_2\alpha^{+/-}$ mice had numerous multifocal to coalescing inflammatory cell infiltrates in the brain and spinal cord (Fig. 3), and all $cPLA_2\alpha^{+/-}$ mice had infiltrates in the brain and spinal cord. Infiltrates were present in the leptomeninges, around blood vessels in the leptomeninges and white matter, and in the parenchyma

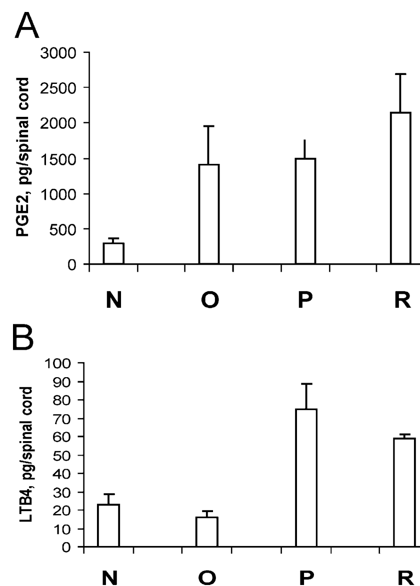

Figure 1. Lipid mediators are increased in spinal cords of mice with EAE. Mice were killed and perfused extensively with PBS. Spinal cords from naive mice (N), or mice with onset (O), peak (P), or recovery (R) of EAE were isolated, extracted with methanol, and levels of PGE2 (A) and LTB4 (B) were measured using ELISA. Levels of PGE2 and LTB4 were increased significantly at peak and recovery of EAE ($P < 0.05$).

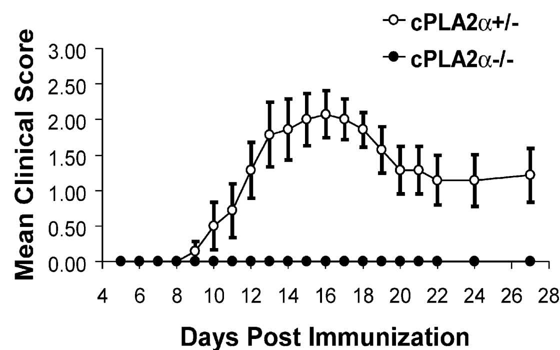

Figure 2. $cPLA_2\alpha^{-/-}$ mice are resistant to EAE induction by immunization with MOG. $cPLA_2\alpha^{-/-}$ or $cPLA_2\alpha^{+/-}$ mice were immunized with MOG/CFA and injected with pertussis toxin on day 0. Paralysis (clinical evidence of EAE) was assessed, starting on day 5 after immunization. Animals were scored as follows: 1, limp tail; 2, partial hind leg paralysis; 3, complete hind leg paralysis or partial hind and front leg paralysis; 4, complete hind and partial front leg paralysis; 5, complete hind and partial or complete front paralysis with severely reduced responsiveness to external stimuli. Data are shown as a mean clinical score ± SE of eight $cPLA_2\alpha^{-/-}$ and seven $cPLA_2\alpha^{+/-}$ mice. The incidence of EAE was 100% for $cPLA_2\alpha^{+/-}$ and 0% for $cPLA_2\alpha^{-/-}$ mice. Data are representative of three independent experiments.

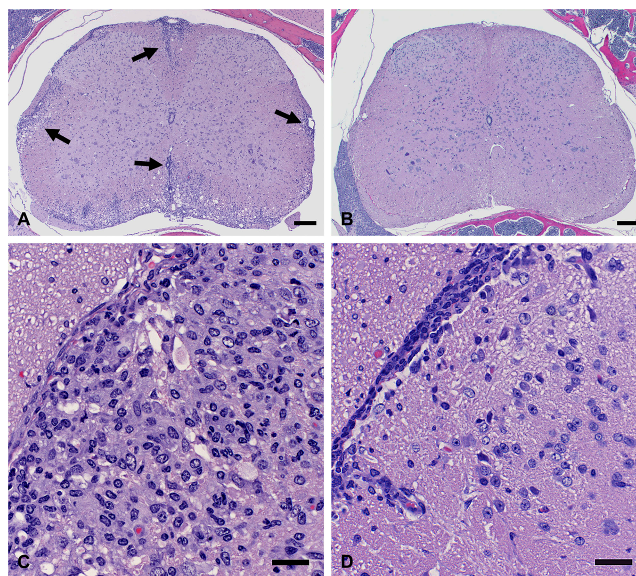

Figure 3. Microscopic changes in transverse sections of spinal cords from $cPLA_2\alpha^{+/-}$ and $cPLA_2\alpha^{-/-}$ mice immunized with MOG/CFA and injected with pertussis toxin. Low magnification of thoracolumbar spinal cord from $cPLA_2\alpha^{+/-}$ (A) and $cPLA_2\alpha^{-/-}$ (B) mice. Bars, 0.2 mm. (A) Multifocal to coalescing inflammation in the leptomeninges, around blood vessels in the leptomeninges and white matter, and parenchyma of the white matter (several areas of inflammation shown by arrows), representative of changes in $cPLA_2\alpha^{+/-}$ mice. There also is vacuolation in the white matter that is consistent with edema. (B) No inflammation in $cPLA_2\alpha^{-/-}$ mouse. Inflammation was absent in 50% of $cPLA_2\alpha^{-/-}$ mice. (C and D). Higher magnification of spinal cords, dorsolateral lumbar region from $cPLA_2\alpha^{+/-}$ (C) and $cPLA_2\alpha^{-/-}$ (D) mice. Bars, 0.05 mm. The spinal nerve is in the upper left. (C). Intense inflammation in the leptomeninges and white matter of the spinal cord, representative of changes in $cPLA_2\alpha^{+/-}$ mice. A few dilated axons also are present. (D) When present, inflammation was much less severe in $cPLA_2\alpha^{-/-}$ mice, and was primarily in the leptomeninges.

of the white matter; in the brain they also were localized around the ventricles. Infiltrates in most animals consisted of mononuclear cells, primarily lymphocytes, macrophages, and glial cells. Occasionally, neutrophils and fewer eosinophils also were observed, although in one $cPLA_2\alpha^{+/-}$ mouse they approached 50% of the infiltrating cells. Pallor and vacuolation were associated with the inflammatory cell infiltrates, which were consistent with edema and demyelination, and dilated axons were observed sometimes. Changes were more common in the posterior sections of spinal cord, compared with the anterior sections. Luxol fast blue stains showed demyelination at the sites of inflammatory cell infiltrates. In contrast, brains from two out of six $cPLA_2\alpha^{-/-}$ mice and spinal cords from three out of six $cPLA_2\alpha^{-/-}$ mice had no infiltrates in any section examined. When present, the changes in $cPLA_2\alpha^{-/-}$ mice were much less severe compared with $cPLA_2\alpha^{+/-}$ mice (Fig. 3). In addition, infiltrates tended to be less common in the parenchyma of the brain and spinal cord of $cPLA_2\alpha^{-/-}$ mice compared with $cPLA_2\alpha^{+/-}$ mice. Similar to $cPLA_2\alpha^{+/-}$ mice, the infiltrates in $cPLA_2\alpha^{-/-}$ mice were primarily mononuclear cells.

To determine if $cPLA_2\alpha^{-/-}$ mice had defective priming of MOG-specific T cells in vivo, we tested antigen-specific proliferation of LN T cells against the immunizing antigen.

Table I. Microscopic changes in CNS of $cPLA_2\alpha^{+/-}$ and $cPLA_2\alpha^{-/-}$ mice immunized with MOG

			Spinal cord					
	Brain		Cervical		Thoracic		Lumbar	
Group	Incidence[a]	Mean no. of foci[b]	Incidence	Mean no. of foci	Incidence	Mean no. of foci	Incidence	Mean no. of foci
	(%)		(%)		(%)		(%)	
$cPLA_2\alpha^{+/-}$	6/6[c] (100)	36.0 ± 4.9	6/6 (100)	26.0 ± 5.7	6/6 (100)	33.0 ± 4.3	6/6 (100)	47.2 ± 23.8
$cPLA_2\alpha^{-/-}$	4/6 (67)	4.8 ± 2.2[d]	1/6 (17)	4.3[e] ± 4.3	3/6 (50)	4.0[d] ± 3.2	3/6 (50)	6.3[e] ± 2.9

[a]Mice with any inflammatory cell infiltrates were considered positive.
[b]Mean number of foci of ≥20 inflammatory cells ± SEM.
[c]Number of mice affected/number of mice examined (%).
[d]$P < 0.0005$ compared with $cPLA_2\alpha^{+/-}$.
[e]$P < 0.005$ compared with $cPLA_2\alpha^{+/-}$.

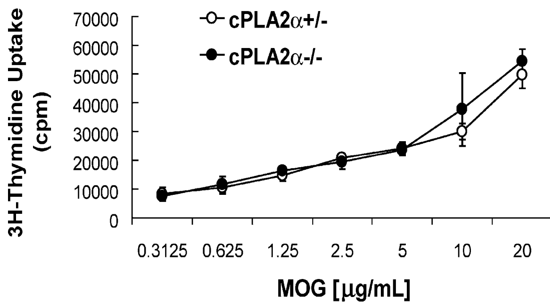

Figure 4. Proliferation of antigen-specific T cells is not impaired in $cPLA_2\alpha^{-/-}$ mice. $cPLA_2\alpha^{-/-}$ or $cPLA_2\alpha^{+/-}$ mice were immunized at the base of the tail with MOG/CFA, and inguinal LNs were collected 10 d later. T cell proliferation was measured in the presence of various concentrations of MOG. Cells were cultured for 48 h and pulsed with [^{3}H]thymidine during the additional 14–18 h of culture. Data are shown as mean ± SD of six wells and are representative of two independent experiments.

When stimulated with a wide range of concentrations of MOG in vitro, cells that were isolated from the draining LNs of the immunized $cPLA_2\alpha^{-/-}$ and $cPLA_2\alpha^{+/-}$ mice proliferated similarly (Fig. 4). The same results were obtained when purified T cells were isolated from the draining LNs and stimulated with MOG in the presence of WT irradiated B6 spleen cells used as APCs (unpublished data).

$cPLA_2\alpha$ plays important roles in the induction and the effector phases of EAE

After establishing that $cPLA_2\alpha^{-/-}$ mice are resistant to EAE development when immunized with MOG, we sought to determine if $cPLA_2\alpha$ plays a role in the induction or effector phases of EAE, or both. To examine these possibilities, we developed a robust EAE adoptive transfer model in B6 mice.

We tested multiple procedures of inducing EAE in B6 mice using an adoptive transfer model, and were able to identify conditions under which robust EAE develops in nearly 100% of the recipient mice (Table S1, available at http://www.jem.org/cgi/content/full/jem.20050665). We were able to generate encephalitogenic cells from spleen and draining LNs of immunized donor animals. To induce EAE, spleen or LN cells from the immunized donors needed to be cultured with exogenous IL-12, and the recipient mice needed to be irradiated sublethally (500 R). EAE was induced with as few as 5.8×10^6 cultured spleen cells. We also isolated $CD4^+$ cells at the end of the culture period; 3 million of these purified $CD4^+$ T cells were able to transfer EAE (unpublished data). In all further adoptive transfer experiments, spleens from the immunized mice were used as a source of encephalitogenic T cells and the recipient mice were irradiated sublethally.

To address whether $cPLA_2\alpha$ plays a role in the effector phase of EAE, we tested the ability of encephalitogenic cells that were generated from immunized WT animals to induce EAE in $cPLA_2\alpha^{-/-}$ or $cPLA_2\alpha^{+/-}$ mice. In three independent experiments, cells that were isolated from WT mice in-

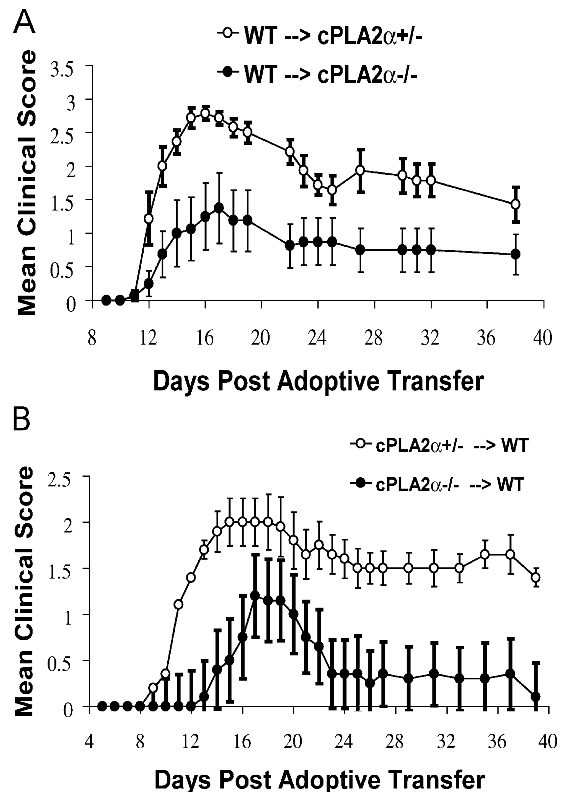

Figure 5. $cPLA_2\alpha$ plays a role in the effector and in the induction phases of EAE. (A) Encephalitogenic cells were prepared by immunizing WT mice and culturing their spleen cells in the presence of MOG and IL-12 for 3 d. 10^7 cells were injected i.p. into sublethally irradiated (500 R) $cPLA_2\alpha^{-/-}$ or $cPLA_2\alpha^{+/-}$ mice. EAE clinical scores were assessed as described in Fig 2. Data are shown as a mean clinical score ± SE of eight $cPLA_2\alpha^{-/-}$ mice and seven $cPLA_2\alpha^{+/-}$ mice. The incidence of EAE in the $cPLA_2\alpha^{+/-}$ group was 100% and was 50% in the $cPLA_2\alpha^{-/-}$ group. The statistical significance of the difference between the curves was determined using ANOVA, $P < 0.001$. Data are representative of three independent experiments. (B) Encephalitogenic cells were prepared by immunizing $cPLA_2\alpha^{-/-}$ or $cPLA_2\alpha^{+/-}$ mice and culturing their spleen cells in the presence of MOG and IL-12 for 3 d. 10^7 cells were injected i.p. into sublethally irradiated (500 R) WT mice. EAE clinical scores were assessed as described in Fig 2. Data are shown as a mean clinical score ± SE of 10 mice per group. The incidence of EAE in mice that were injected with $cPLA_2\alpha^{+/-}$ cells was 70%; it was 90% in mice that were injected with $cPLA_2\alpha^{-/-}$ cells. The statistical significance of the difference between the curves was determined using ANOVA, $P < 0.0001$. Data shown are representative of two independent experiments.

duced less severe EAE in $cPLA_2\alpha^{-/-}$ mice than in $cPLA_2\alpha^{+/-}$ control mice (Fig. 5 A). The day of onset of EAE was the same in both groups of recipient mice. These results indicate that $cPLA_2\alpha$ plays a role in the effector phase of EAE. However, because a complete absence of clinical EAE never was observed in $cPLA_2\alpha^{-/-}$ mice that received encephalitogenic cells from WT animals, we tested the possibility that $cPLA_2\alpha$ also plays a role in the induction of the encephalitogenic immune responses. We immunized $cPLA_2\alpha^{-/-}$ or $cPLA_2\alpha^{+/-}$ mice with MOG, and used the cells that were derived from

these mice to induce EAE in WT recipients. Cells that were isolated from cPLA$_2\alpha^{-/-}$ and cPLA$_2\alpha^{+/-}$ mice were able to induce EAE in the WT recipients. However, cells that originated from cPLA$_2\alpha^{-/-}$ mice induced less severe EAE with delayed onset (Fig. 5 B). These results suggest that, in addition to playing a role in the effector phase, cPLA$_2\alpha$ plays a role in the induction phase of the disease.

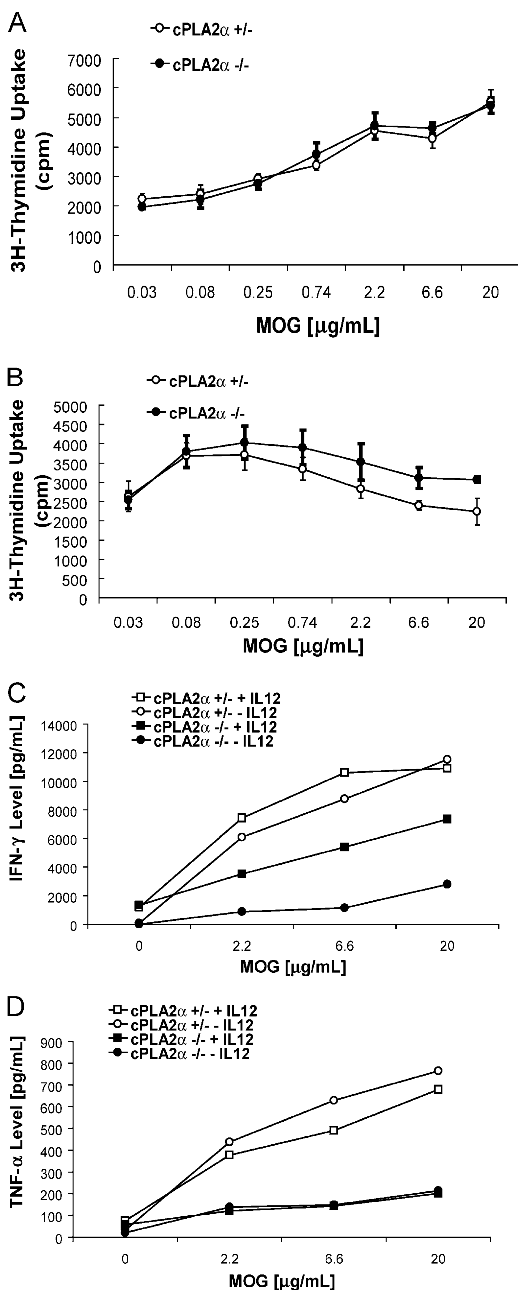

Figure 6. Proliferation and cytokine production by splenic cells from immunized cPLA$_2\alpha^{-/-}$ and cPLA$_2\alpha^{+/-}$ mice stimulated with MOG. The mice were immunized at two sites s.c. with MOG/CFA, and spleens were collected 10 d later. T cell stimulation was set up in the presence of various concentrations of MOG and in the (A) absence or (B) presence of 30 ng/ml IL-12. Cells were cultured for 62–72 h and pulsed with [^{3}H]thymidine during the last 14–18 h of culture. Proliferation data are shown as a mean cpm $\pm$ SD of six wells. Supernatants were collected after 48 h of culture and the amounts of (C) IFN-γ and (D) TNF were determined in the pools of supernatants of six wells. Data shown are representative of two independent experiments.

Development of Th1-type responses is impaired in cPLA$_2\alpha^{-/-}$ mice

To assess why cells from the immunized cPLA$_2\alpha^{-/-}$ mice did not induce EAE that was as severe as that observed with cells from cPLA$_2\alpha^{+/-}$ controls, we tested antigen-specific proliferation of these effector cells. In the presence of various amounts of MOG, spleen cells that were isolated from cPLA$_2\alpha^{-/-}$ and cPLA$_2\alpha^{+/-}$ control animals proliferated similarly (Fig. 6 A). The proliferation of both types of cells was reduced in the presence of IL-12, but cells from cPLA$_2\alpha^{-/-}$ and cPLA$_2\alpha^{+/-}$ animals proliferated similarly (Fig. 6 B). However, when we measured the amounts of

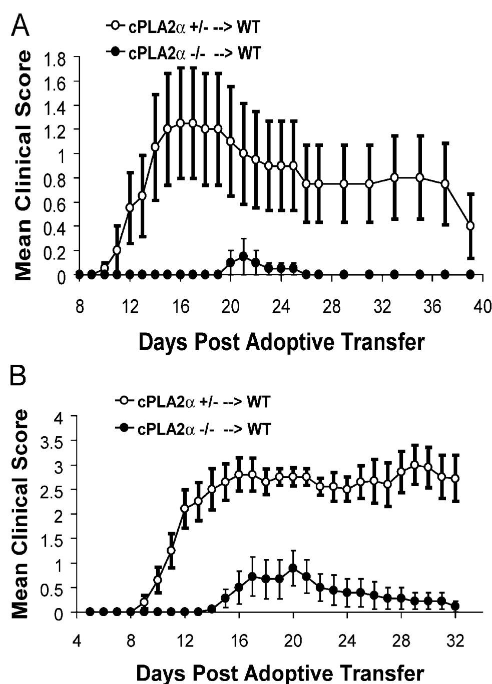

Figure 7. Absence of IL-12 during the culture period impaired the ability of T cells from cPLA$_2\alpha^{-/-}$ mice to induce EAE. Encephalitogenic cells were prepared by immunizing cPLA$_2\alpha^{-/-}$ or cPLA$_2\alpha^{+/-}$ mice and culturing their spleen cells in the presence of MOG without addition of exogenous IL-12. In two independent experiments, (A) 10^7 or (B) 4.5×10^7 cells were injected i.p. into sublethally irradiated (500 R) WT mice. EAE clinical scores were assessed as described in Fig 2. (A) Data are shown as a mean clinical score $\pm$ SE of 10 mice/group. The incidence of EAE in mice that were injected with cPLA$_2\alpha^{+/-}$ cells was 50%; it was 10% in mice that were injected with cPLA$_2\alpha^{-/-}$ cells. The statistical significance of the difference between the curves was determined using ANOVA, $P < 0.0001$. (B) Data are shown as a mean clinical score $\pm$ SE of 10 cPLA$_2\alpha^{+/-}$ mice and 9 cPLA$_2\alpha^{-/-}$ mice. The incidence of EAE in mice that were injected with cPLA$_2\alpha$+/− cells was 100%; it was 50% in mice that were injected with cPLA$_2\alpha^{-/-}$ cells. The statistical significance of the difference between the curves was determined using analysis of variance, $P < 0.0001$.

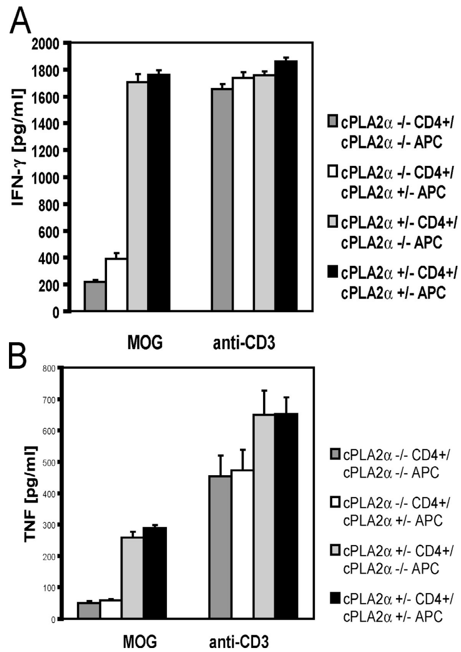

Figure 8. The defect in production of Th1-type cytokines in cells from cPLA$_2\alpha^{-/-}$ is acquired during in vivo priming. CD4$^+$ cells and non-CD4$^+$ cells (APCs) were isolated from the draining LNs of cPLA$_2\alpha^{-/-}$ or cPLA$_2\alpha^{+/-}$ mice that were immunized previously with MOG/CFA. CD4$^+$ cells and APCs from the two types of mice were mixed and the ability of cells in these cultures to produce (A) IFN-γ or (B) TNF was measured after stimulation with MOG (20 μg/ml) or anti-CD3 (1 μg/ml).

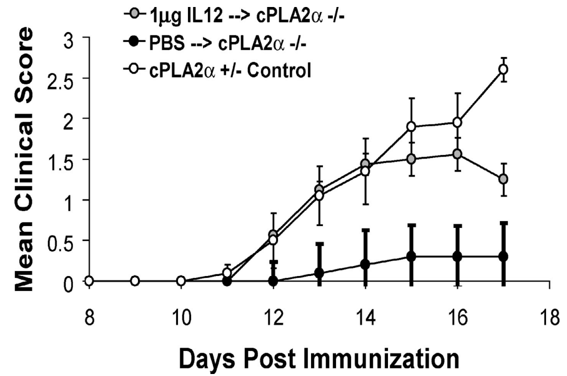

Figure 9. Administration of IL-12 renders cPLA$_2\alpha^{-/-}$ mice susceptible to EAE. cPLA$_2\alpha^{-/-}$ or cPLA$_2\alpha^{+/-}$ mice were immunized with MOG/CFA and injected with pertussis toxin on day 0. Then cPLA$_2\alpha^{-/-}$ mice received PBS or IL-12 (0.1 mg/dose) i.p. on days 1, 3, 5, 7, and 9. EAE clinical scores were assessed as described in Fig 1. Data are shown as a mean clinical score ± SE of 10 cPLA$_2\alpha^{-/-}$ mice that were treated with PBS, 8 cPLA$_2\alpha^{-/-}$ mice that were treated with IL-12, and 10 untreated cPLA$_2\alpha^{+/-}$ mice. The incidence of EAE was 20% for PBS-treated cPLA$_2\alpha^{-/-}$ mice, 75% for IL-12–treated cPLA$_2\alpha^{-/-}$ mice, and 100% for cPLA$_2\alpha^{+/-}$ mice. The statistical significance of the difference between the curves for PBS-treated cPLA$_2\alpha^{-/-}$ mice versus IL-12–treated cPLA$_2\alpha^{-/-}$ mice was determined using ANOVA, $P < 0.0001$. There was no statistically significant difference between the curves for IL-12–treated cPLA$_2\alpha^{-/-}$ mice versus cPLA$_2\alpha^{+/-}$ mice as determined using analysis of variance, $P = 0.416$.

various cytokines in these MOG-stimulated cultures, a striking reduction in IFN-γ levels was observed in cultures of cPLA$_2\alpha^{-/-}$ cells when compared with cPLA$_2\alpha^{+/-}$ controls (Fig. 6 C). These reduced IFN-γ levels could be restored, in part, by adding IL-12 to the cultures (Fig. 6 C). In addition, levels of TNF, another Th1 cytokine, were reduced dramatically in cPLA$_2\alpha^{-/-}$ cultures in comparison with the controls. TNF production was not restored upon addition of IL-12 to the cultures (Fig. 6 D). As an indication that there was no skewing toward Th2-type responses, there were no detectable levels of IL-4 or -5 in cPLA$_2\alpha^{-/-}$ or cPLA$_2\alpha^{+/-}$ cultures (unpublished data).

To test a potential role of exogenously added IL-12 during the in vitro generation of encephalitogenic cells from cPLA$_2\alpha^{-/-}$ mice, we performed an adoptive transfer experiment in which no IL-12 was added during the culture period. When EAE was induced using cells that were cultured in the absence of exogenously added IL-12, an even greater difference in the ability of cells from cPLA$_2\alpha^{-/-}$ and cPLA$_2\alpha^{+/-}$ mice to induce EAE was observed. When 10^7 encephalitogenic cells were used to induce EAE, the cells that originated from cPLA$_2\alpha^{+/-}$ mice induced mild clinical EAE, whereas cells from cPLA$_2\alpha^{-/-}$ mice induced almost no clinical EAE (Fig. 7 A). When higher numbers of encephalitogenic cells were used (4.5×10^7) to induce EAE, cPLA$_2\alpha^{+/-}$ cells induced severe disease, whereas cells from cPLA$_2\alpha^{-/-}$ mice induced mild disease with a delayed onset (Fig. 7 B). These results indicate that when IL-12 is added to the cultures of MOG-specific T cells, it contributes to the generation of encephalitogenic cells and may mask endogenous defect in their development.

We next tested if T cells that were isolated from cPLA2$\alpha^{-/-}$ mice have an intrinsic defect in producing Th1-type cytokines or if this defect was acquired during the priming in vivo. We separated CD4$^+$ and non-CD4$^+$ cells (designated APCs thereafter) from the draining LNs of MOG-immunized cPLA2$\alpha^{-/-}$ and cPLA2$\alpha^{+/-}$ mice. When CD4$^+$ cells and APC cells were from cPLA2$\alpha^{-/-}$ mice, stimulation by MOG produced eight- and fivefold lower levels of IFN-γ and TNF, respectively, compared with the levels that were produced in co-cultures of the cells from cPLA2$\alpha^{+/-}$ mice (Fig. 8). Much of this difference was maintained when CD4$^+$ cells from cPLA2$\alpha^{+/-}$ and cPLA2$\alpha^{-/-}$ mice were stimulated with APCs from the cPLA2$\alpha^{+/-}$ mice. However, the source of APCs did have an effect on IFN-γ levels when CD4$^+$ cells from cPLA2$\alpha^{-/-}$ mice were stimulated with MOG and APCs from cPLA2$\alpha^{+/-}$ mice. The levels of IFN-γ produced were almost twice as high as the levels that were produced in cultures in which APCs were from cPLA2$\alpha^{-/-}$ mice. Changing the source of APCs had no effect on the levels of TNF that were produced in these cultures. To test if the defect in Th1 cytokine production was limited to MOG-specific T cells or was attributed to of all T cells from cPLA2$\alpha^{+/-}$ mice, the cultures were stimulated with anti-CD3 mAb's. In these cultures, T

cells from cPLA2$\alpha^{-/-}$ and cPLA2$\alpha^{+/-}$ mice produced similar levels of IFN-γ, regardless of the source of the APCs. Amounts of TNF produced by cPLA2$\alpha^{-/-}$ CD4 cells were lower (~30%) than amounts of TNF that were produced by CD4$^+$ cells from cPLA2$\alpha^{+/-}$ mice. This indicated that an intrinsic defect in production of TNF may exist in cells from cPLA2$\alpha^{-/-}$ mice. However, this defect is much less pronounced than the fivefold defect in TNF that was observed for MOG-specific T cells from cPLA2$\alpha^{-/-}$ mice.

Injection of IL-12 during disease induction period renders cPLA$_2$$^{-/-}$ mice susceptible to EAE

The data obtained in the above-described studies indicated that Th1 differentiation is impaired in cPLA$_2\alpha^{-/-}$ animals. Therefore, we tested whether injection of recombinant mouse IL-12, a potent inducer of Th1 differentiation, during the immunization phase would render cPLA$_2\alpha^{-/-}$ mice susceptible to EAE. When cPLA$_2\alpha^{-/-}$ mice were immunized with MOG/CFA and injected with 0.1 μg recombinant murine IL-12 on the day of immunization and every other day after that for a total of five injections, most of the cPLA$_2\alpha^{-/-}$ mice developed EAE (Fig. 9).

DISCUSSION

In the present study, we demonstrate that cPLA$_2\alpha$ is essential for the development of EAE. cPLA$_2\alpha$ plays a role in the induction of the encephalitogenic immune responses and in the effector phase of EAE. cPLA$_2\alpha$ plays an important role in enhancing the production of Th1-type cytokines. The defect in Th1-type cytokine production and the reduced encephalitogenic potential of cPLA$_2\alpha^{-/-}$ cells can be reversed, in part, by providing exogenous IL-12. Similarly, in vivo administration of IL-12 during the induction phase of the disease can render cPLA$_2\alpha^{-/-}$ mice susceptible to EAE.

During the course of EAE development, we detected significant increases in the levels of PGE_2 and LTB_4, which were indicative of PLA_2 activation during the peak and recovery of the disease. This is consistent with the published observation that the levels of cPLA$_2\alpha$ are increased during EAE development (22), and with the observation that these mediators are increased in CSF of patients who have MS (17).

When immunized with MOG, cPLA$_2\alpha^{-/-}$ mice were almost completely resistant to EAE induction. Our finding is consistent with the previously published report that treatment of MOG-immunized mice with AACOCF3, which inhibits cPLA$_2\alpha$, inhibits development of EAE in a dose-dependent manner (22). The observed clinical resistance to EAE development was confirmed by microscopic examination of the brains and spinal cords, which revealed a great reduction in the number of inflammatory foci in the CNS tissues from the immunized cPLA$_2\alpha^{-/-}$ mice, compared with cPLA$_2\alpha^{+/-}$ mice. However, several immunized cPLA$_2\alpha^{-/-}$ mice had reduced, but detectable, inflammation in CNS in the absence of clinical signs of EAE. Such clinically silent inflammatory lesions have been described in MOG-induced EAE in mice and rats (37, 38), and clinically silent MRI lesions are common in patients who have MS (39). It has been suggested that the size and location of the lesions, as well as their ability to recruit macrophage/microglia may be related to the clinical silence of the lesions (37–39). The presence of these inflammatory lesions in ~50% of cPLA$_2\alpha^{-/-}$ mice demonstrates that MOG-specific T cells that are capable of entering the CNS were generated in cPLA$_2\alpha^{-/-}$ mice. There are at least two possible explanations for the lack of clinical signs in these mice. First, it is possible that in cPLA$_2\alpha^{-/-}$ mice, the number of encephalitogenic cells or their function is not adequate for a full development of EAE. In this case, the induction phase of EAE would be deficient in cPLA$_2\alpha^{-/-}$ mice. Second, it is possible that encephalitogenic T cells, once in the CNS, are not able to mediate full EAE development. In this latter case, resistance to EAE induction in cPLA$_2\alpha^{-/-}$ mice would be a result of a defective effector phase of EAE. It is well-established that encephalitogenic T cells represent only a small fraction of the cells in the inflammatory lesions, whereas most of the cells are recruited to the site in an antigen-independent manner (25, 40).

To determine whether cPLA$_2\alpha$ plays a role in the induction or the effector phase of EAE, we used the adoptive transfer model of the disease. Transfer of encephalitogenic cells that were generated from immunized WT donors into cPLA$_2\alpha^{-/-}$ recipients resulted in EAE with the same time of onset, but lesser severity, compared with transfer of the same cells into cPLA$_2\alpha^{+/-}$ recipients. This indicates that cPLA$_2\alpha$ plays a role in the effector phase of EAE, and is consistent with the previous report that cPLA$_2\alpha$ is expressed by endothelial cells and macrophages as well as CD4$^+$ T cells in the EAE lesions (22). Multiple downstream products of cPLA$_2\alpha$ enzymatic activity may play a role in enhancing and sustaining inflammatory responses in CNS during the effector phase of EAE. For example, LTB_4 is a potent chemoattractant of myeloid cells as well as CD4 and CD8 effector cells (41–44). In addition, LTs enhance phagocytic capacity and the generation of other proinflammatory mediators by leukocytes (45). In addition to generating AA, cPLA$_2\alpha$ also generates the lysophospholipid precursor of PAF—which may play an important role during the effector phase of EAE—by acting as a strong chemoattractant and by increasing the permeability of blood–brain barrier, and thereby, facilitating entry of proinflammatory cells into CNS (24, 46). Recent work by Kihara et al. (47) showed that PAF receptor–deficient mice have reduced incidence and severity of EAE.

Because active immunization of cPLA$_2\alpha^{-/-}$ mice resulted in almost no clinical EAE, whereas the adoptive transfer of encephalitogenic cells into cPLA$_2\alpha^{-/-}$ mice resulted in mild but significant EAE, we considered the possibility that cPLA$_2\alpha$ also plays a role in the induction of encephalitogenic immune responses. We compared EAE development in WT recipient mice that were injected with encephalitogenic cells that were generated from MOG-immunized cPLA$_2\alpha^{-/-}$ or cPLA$_2\alpha^{+/-}$ mice. EAE that was induced by cPLA$_2\alpha^{-/-}$ cells had a delayed onset and lower severity compared with EAE that was induced by control cells; this

showed that $cPLA_2\alpha$ also plays a role in the induction of encephalitogenic immune response.

The reduced encephalitogenic potential of the $cPLA_2\alpha^{-/-}$ cells coincided with reduced production of IFN-γ and TNF by these cells in vitro without an increase in Th2-type cytokines. At the same time, there was no reduction in MOG-stimulated proliferation of T cells from immunized $cPLA_2\alpha^{-/-}$ mice, which indicated that priming and expansion of MOG-specific T cells was not affected by the defect in $cPLA_2\alpha$. The defective production of Th1-type cytokines could be restored, in part, by the addition of IL-12 to the cultures. Addition of IL-12 also enhanced the encephalitogenic potential of cells that were isolated from $cPLA_2\alpha^{-/-}$ mice. When IL-12 was omitted from the cultures, cells that were isolated from $cPLA_2\alpha^{-/-}$ mice had an even lower ability to induce EAE. Conversely, in vivo administration of IL-12 during the immunization period restored the ability of these mice to develop EAE, which further suggests that at least part of the defect in developing EAE in these mice may be related to the defective differentiation toward Th1-type responses. Our preliminary experiments have shown that adoptive transfer of up to 25 million $CD4^+$ T cells from $cPLA_2\alpha^{+/-}$ mice into $cPLA_2\alpha^{-/-}$ mice does not render $cPLA_2\alpha^{-/-}$ mice susceptible to EAE. Further experiments are required to determine which cells need to produce cPLA2α for Th1-type responses to develop. Our results indicate that $CD4^+$ cells from $cPLA_2\alpha^{-/-}$ mice do not have an intrinsic defect in the production of Th1-type cytokines. Instead, the defect was acquired during T cell priming in vivo.

The mechanism by which $cPLA_2\alpha$-mediated AA release may enhance development of Th1 responses is not known. $cPLA_2\alpha$-mediated AA release was shown to be critical in MCP-1–, MCP-3–, regulated on activation, normal T cell expressed and secreted–, and monocyte inflammatory protein-1α–mediated activation and migration of human monocytes (48); therefore, it is possible that macrophage recruitment and/or activation is impaired in $cPLA_2\alpha^{-/-}$ mice. Macrophages are believed to be a major effector cell in the EAE inflammatory response, and the severity of EAE correlates with the number of macrophages detected in the CSF of mice that have EAE (25, 49, 50). Mice that are deficient in MCP-1 or its receptor, CCR2, have reduced numbers of macrophage infiltrates and are resistant to EAE development when injected with WT encephalitogenic T cells (51, 52). It is noteworthy that serum levels of IFN-γ were reduced significantly in MCP-$1^{-/-}$ mice at the time of peak disease, which indicated an impaired Th1 response that was analogous to that seen in the $cPLA_2\alpha^{-/-}$ mice (52). Unlike cells that were isolated from $cPLA_2\alpha^{-/-}$ mice, MCP-$1^{-/-}$ T cells were fully encephalitogenic when transferred to WT mice. However, as the investigators noted, IL-12 that was present during generation of these encephalitogenic cells may have masked the defect in Th1 differentiation (52). Thus, it is possible that in the absence of $cPLA_2\alpha$, MCP-1/CCR2-mediated macrophage trafficking and/or activation is impaired, which results in reduced tissue injury and insufficient development or maintenance of Th1 effector cell function.

Products of $cPLA_2\alpha$ also may play a role in Th1/Th2 differentiation. Although it is possible that LTs also regulate skewing of Th1/Th2 cells, we do not believe that LTs are critical for initial skewing toward Th1 responses in the EAE model, because 5-LO–deficient mice develop EAE with a similar time of onset and increased severity when compared with control mice (53). Conflicting data exist on the possible role of PG in IL-12 induction. For example, it was demonstrated that exposure of bone marrow–derived immature DCs to PGE_2 suppresses IL-12 production by these cells by way of increased IL-10 production (54) which would lead to decreased Th1 differentiation. In contrast, PGE_2 was demonstrated to enhance IL-12 production when immature DCs were stimulated with TNF (55). It is possible that multiple lipid mediators can influence Th1/Th2 cell differentiation by affecting multiple cell types. The effects of these mediators may be influenced further by the presence of other inflammatory mediators at the site of inflammation, and it is possible that the same lipid mediator may have opposite effects under different in vivo conditions. It also is possible that $cPLA_2\alpha^{-/-}$ mice have deficient Th1 responses because of reduced IL-23 production. PGE_2 and pharmacologic agonists of the prostaglandin E_2 receptor (EP)2 and EP2/EP4 receptors recently were shown to induce IL-23 production from unstimulated immature DCs (56). $cPLA_2\alpha$-deficient mice recently were shown to be resistant to induction of collagen-induced rheumatoid arthritis, another autoimmune disease that is believed to be mediated primarily by Th1 responses, in which macrophages are believed to be major cells that mediate the autoimmune pathology (57).

In conclusion, we demonstrated that $cPLA_2\alpha$ is necessary for EAE development and that this enzyme plays a role during the induction and the effector phases of EAE. In the absence of $cPLA_2\alpha$, induction of Th1-type responses is impaired; we suggest that $cPLA_2\alpha$ may represent a novel therapeutic target for treatment of MS and other Th1-mediated diseases.

MATERIALS AND METHODS

Mice. $cPLA_2\alpha^{-/-}$ mice on C57BL/6 background were generated by backcrossing $cPLA_2\alpha^{-/-}$ mice of mixed B6/129 background (21) to B6 background for 12 generations and then intercrossing $cPLA_2\alpha^{+/-}$ mice to obtain $cPLA_2\alpha$WT, $cPLA_2\alpha^{+/-}$, and $cPLA_2\alpha^{-/-}$ mice, or crossing $cPLA_2\alpha^{+/-}$ and $cPLA_2\alpha^{-/-}$ mice to obtain $cPLA_2\alpha^{+/-}$ and $cPLA_2\alpha^{-/-}$ littermates. All $cPLA_2\alpha$ mice were bred at Taconic Farms and were used at 6–10 wk of age. In all experiments, age- and sex-matched littermates were used to compare $cPLA_2\alpha^{-/-}$ with $cPLA_2\alpha^{+/-}$ and/or $cPLA_2\alpha$WT mice. For some experiments, WT female C57BL/6 mice were purchased from Jackson ImmunoResearch Laboratories or Taconic Farms and used at 6–10 wk of age. These mice were used only when no direct comparison was done to $cPLA_2\alpha^{-/-}$ mice. Wyeth Institutional Animal Care and Use Committee was the overseeing body that approved the animal studies.

EAE induction. For EAE induction using immunization with MOG, all mice were injected s.c. at two sites with a total of 200 μg of MOG peptide 35–55 in CFA containing 6 mg/ml killed *Mycobacterium tuberculosis*. On the same day, the mice received 500 ng pertussis toxin (List Laboratories) i.p. For

EAE induction in the adoptive transfer model, recipient mice were irradiated sublethally (500 R) and within 16 h were injected i.p. with encephalitogenic cells, prepared as described below. Paralysis (clinical evidence of EAE) was assessed daily, starting on day 5 after immunization or adoptive transfer, when all the mice were still clinically normal. Clinically, animals were scored as follows: 1, limp tail; 2, partial hind leg paralysis; 3, complete hind leg paralysis or partial hind and front leg paralysis; 4, complete hind and partial front leg paralysis; 5, complete hind and partial or complete front paralysis with severely reduced responsiveness to external stimuli. Mice were killed immediately if they scored 5, or if they scored 4 on two consecutive days.

Preparation of cells for EAE induction in adoptive transfer model. To prepare MOG-specific cells that were able to induce EAE in the adoptive transfer model, mice were immunized with MOG/CFA in the same fashion as when inducing EAE, but no pertussis toxin was administered. Spleens were collected 10 d later, single-cell suspension was prepared and red blood cells were lysed. Stimulation with MOG was performed in T150 flasks, using 6×10^6 cells/ml and 20 μg MOG_{35-55}/ml in T cell medium (RPMI media enriched with 10% FBS, 2 mM L-glutamine, 5×10^{-5} M 2-ME, nonessential amino acids, sodium pyruvate, and penicillin/streptomycin). Recombinant mouse IL-12 was added to some cultures at 30 ng/ml. 3 d after initiation of the cultures, cells were harvested, washed in PBS, and injected into recipient mice as described above for EAE induction.

Histology. Mice were killed using CO_2 asphyxia and immediately perfused with 10–20 ml of cold PBS. The head and spine were dissected in one piece, and a hole was made in the skull to allow formalin to enter the subarachnoid space. The tissue was immersed into 10% buffered formalin and fixed for at least 72 h before processing. After fixation, brains were removed from the skull before sectioning. Spinal columns with spinal cords were decalcified before sectioning in situ. Four transverse sections of the brain (frontal cortex to medulla), and multiple (n = 9 to 13 sections/animal) transverse sections of the cervical, thoracic, and lumber spinal cord were prepared routinely and stained with hematoxylin and eosin. Spinal cord sections also were stained with Luxol fast blue. The number of inflammatory foci that contained at least 20 cells were counted in each hematoxylin and eosin–stained section in a blinded fashion by the same pathologist (MWL) (58, 59). When foci were coalescing, estimates were made of the number of foci. The presence of vacuolation and pallor in the white matter also were noted. Demyelination was assessed on Luxol fast blue sections.

Determination of PGE_2 and LTB_4 mediators in mouse spinal cords. At different time points during EAE development, mice were killed and extensively perfused with cold PBS (at least 20 ml/mouse). The spinal cords were collected from the animals (two to six spinal cords were pooled together, at least three pools were collected at each time point) and immediately homogenized in methanol. The mediators in the tissues were extracted in 5 ml methanol for 3–5 d at −80°C. To remove the tissue residues, the extracts were centrifuged at 1,800 g for 20 min at 4°C. Then the sample solutions were dried to remove methanol. The dried samples were suspended in an assay buffer that was supplied with each ELISA kit (Assay Designs). To determine the levels of PGE_2 and LTB_4 within the sensitive, (i.e., linear) ranges of the standard curves, the samples were diluted further 1–20 fold and assayed at multiple dilutions.

T cell proliferation and cytokine production analysis. To assess draining LN T cell proliferation and cytokine production against MOG, we immunized mice at the base of the tail with MOG/CFA (using the same amount of MOG and CFA as in EAE induction protocol) and collected inguinal LNs 10 d later. Single-cell suspensions were prepared, and in some experiments, $CD4^+$ and non-$CD4^+$ (APC) cells were isolated using Dynal beads (Invitrogen), according to the manufacturer's instructions. When $CD4^+$ and non-$CD4^+$ cells were isolated, they were mixed at 1:1 ratio. 8×10^5 cells/well were cultured in 96-well plates in T cell medium and various amounts of MOG peptide or anti-CD3 antibodies. 48–54 h after the initiation, the culture supernatants were collected for cytokine analysis and the cultures were pulsed with 0.5 μCi of [^{3}H]thymidine/well and harvested 14–18 h later.

To assess proliferation and cytokine production of splenic T cells against MOG, mice were immunized with MOG/CFA in the same fashion as when inducing EAE, but no pertussis toxin was administered. Spleens were collected 10 d later, single-cell suspensions were prepared, and red blood cells were lysed. Stimulation with MOG was performed as described above for LN cells. Culture supernatants were collected 48 h later for cytokine analysis and cultures were pulsed to determine [^{3}H]thymidine incorporation. Concentrations of IL-4, IL-5, IFN-γ, and TNF in the supernatants were quantified using a cytometric bead array kit obtained from BD Biosciences.

Statistical analysis. For statistical analysis, a Poisson distribution was used to model the inflammatory foci parameter. A square root transformation was applied to stabilize the variance, and then the transformed data were analyzed with a one-way analysis of variance. Severity scores were analyzed using the mean score Mantel-Haenszel statistic. Clinical scores were compared using analysis of variance. Statistical significance of differences in cytokine production and lipid mediator presence in spinal cords was determined using Student's t test.

Online supplemental material. Table S1 shows that effector cells from spleen or lymph node induce EAE in sublethally irradiated, but not in nonirradiated, recipient mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050665/DC1.

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