

# Cytosolic phospholipase A<sub>2</sub>α-deficient mice are resistant to experimental autoimmune encephalomyelitis

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

<sup>1</sup>Department of Inflammation, Wyeth Research, Cambridge, MA 02140

<sup>2</sup>Exploratory Drug Safety, Wyeth Research, Andover, MA 01810

<sup>3</sup>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<sub>2</sub>α (cPLA<sub>2</sub>α), 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<sub>2</sub>α<sup>-/-</sup> mice are resistant to EAE. Histologic examination of the CNS from MOG-immunized mice revealed extensive inflammatory lesions in the cPLA<sub>2</sub>α<sup>+/-</sup> mice, whereas the lesions in cPLA<sub>2</sub>α<sup>-/-</sup> mice were reduced greatly or completely absent. MOG-specific T cells generated from WT mice induced less severe EAE in cPLA<sub>2</sub>α<sup>-/-</sup> mice compared with cPLA<sub>2</sub>α<sup>+/-</sup> mice, which indicates that cPLA<sub>2</sub>α plays a role in the effector phase of EAE. Additionally, MOG-specific T cells from cPLA<sub>2</sub>α<sup>-/-</sup> 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<sub>2</sub>α also plays a role in the induction phase of EAE. MOG-specific T cells from cPLA<sub>2</sub>α<sup>-/-</sup> mice were deficient in production of Th1-type cytokines. Consistent with this deficiency, *in vivo* administration of IL-12 rendered cPLA<sub>2</sub>α<sup>-/-</sup> mice susceptible to EAE. Our data indicate that cPLA<sub>2</sub>α 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; AACOCF<sub>3</sub>, arachidonyl trifluoromethylketone; CNS, central nervous system; cPLA<sub>2</sub>α, cytosolic phospholipase A<sub>2</sub>α; 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<sup>+</sup> 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 immuno-

competent 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.

The online version of this article contains supplemental material.

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<sub>2</sub>α (cPLA<sub>2</sub>α) initiates the production of these inflammatory mediators (20, 21); is expressed by endothelial cells, CD4<sup>+</sup> 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<sub>2</sub>α, 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<sub>4</sub> 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<sub>2</sub>α enzymatic activity in EAE development are not well understood, but Kalyvas and David (22), who documented the presence of cPLA<sub>2</sub>α at the site of lesions, also demonstrated that arachidonyl trifluoromethylketone (AACOCF<sub>3</sub>)—which inactivates cPLA<sub>2</sub>α by reversible reaction with the active site serine (32)—can reduce EAE severity in myelin oligodendrocyte glycoprotein (MOG)-immunized mice. AACOCF<sub>3</sub> was effective at blocking the onset of disease, and, in some cases, preventing relapse. However, AACOCF<sub>3</sub> also inhibits calcium-independent phospholipase A<sub>2</sub> (33), and thromboxane synthase, which is downstream of cyclooxygenase (32). AACOCF<sub>3</sub> 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, AACOCF<sub>3</sub> may be acting on multiple enzymes, and therefore, its effect in EAE may not be due solely to inhibition of cPLA<sub>2</sub>α.

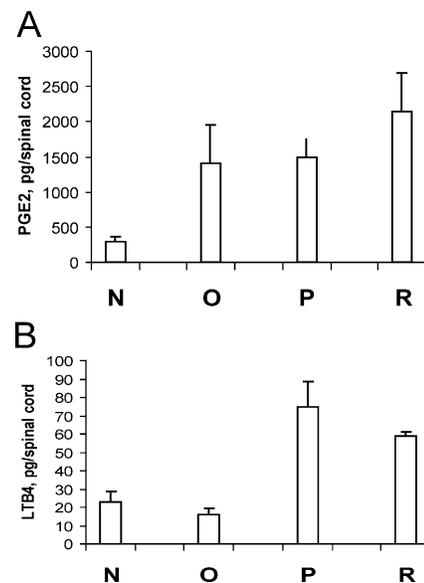
To assess the role of cPLA<sub>2</sub>α in EAE, we established the cPLA<sub>2</sub>α<sup>-/-</sup> mice on the susceptible B6 background, and studied the development of disease in cPLA<sub>2</sub>α<sup>-/-</sup> and cPLA<sub>2</sub>α<sup>+/-</sup> littermates. We found that cPLA<sub>2</sub>α<sup>-/-</sup> 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

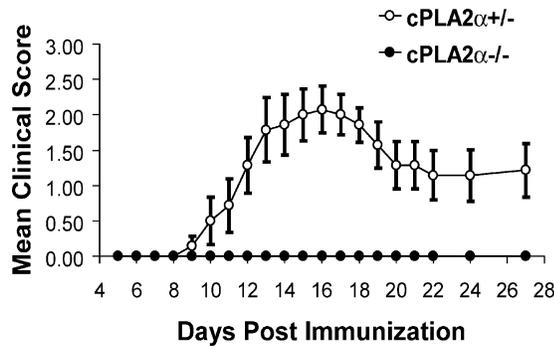
### cPLA<sub>2</sub>α<sup>-/-</sup> mice do not develop EAE after immunization with MOG

To test if end-products of cPLA<sub>2</sub>α 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<sub>2</sub> and LTB<sub>4</sub> 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<sub>2</sub>α in EAE development, we immunized cPLA<sub>2</sub>α<sup>-/-</sup> or cPLA<sub>2</sub>α<sup>+/-</sup> littermates with MOG and followed EAE development. In three independent experiments, cPLA<sub>2</sub>α<sup>-/-</sup> mice were resistant to EAE induction (3 out of 27 mice developed EAE), whereas cPLA<sub>2</sub>α<sup>+/-</sup> mice developed severe EAE (22 out of 23 mice developed EAE) (Fig. 2). There was no difference in the severity of EAE between cPLA<sub>2</sub>α<sup>+/-</sup> and cPLA<sub>2</sub>α<sup>+/+</sup> littermates (unpublished data); therefore, the cPLA<sub>2</sub>α<sup>+/-</sup> littermates were used as controls for cPLA<sub>2</sub>α<sup>-/-</sup> mice in all further experiments. Microscopic examination of the brain and spinal cord showed remarkable differences between the cPLA<sub>2</sub>α<sup>+/-</sup> and cPLA<sub>2</sub>α<sup>-/-</sup> mice (Fig. 3; Table I). The cPLA<sub>2</sub>α<sup>+/-</sup> mice had numerous multifocal to coalescing inflammatory cell infiltrates in the brain and spinal cord (Fig. 3), and all cPLA<sub>2</sub>α<sup>+/-</sup> 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

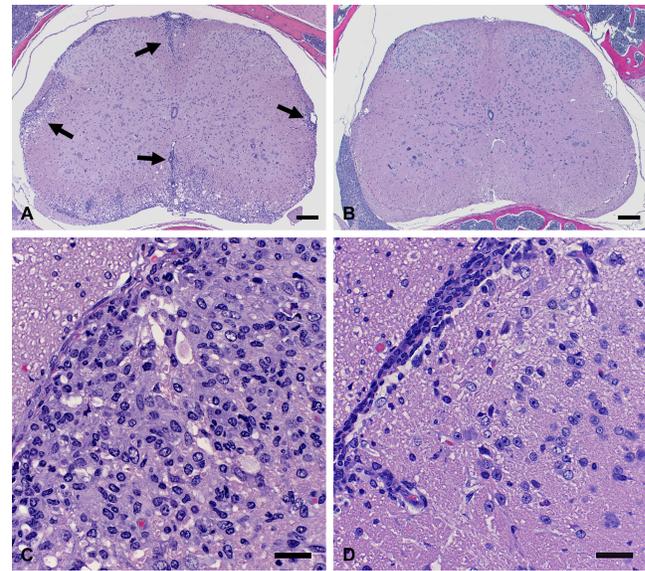


**Figure 1. Lipid mediators are increased in spinal cords of mice with EAE.** Mice were killed and perfused extensively with PBS. Spinal cords from naive mice (N), or mice with onset (O), peak (P), or recovery (R) of EAE were isolated, extracted with methanol, and levels of PGE<sub>2</sub> (A) and LTB<sub>4</sub> (B) were measured using ELISA. Levels of PGE<sub>2</sub> and LTB<sub>4</sub> were increased significantly at peak and recovery of EAE ( $P < 0.05$ ).



**Figure 2.** cPLA<sub>2</sub>α<sup>-/-</sup> mice are resistant to EAE induction by immunization with MOG. cPLA<sub>2</sub>α<sup>-/-</sup> or cPLA<sub>2</sub>α<sup>+/-</sup> 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<sub>2</sub>α<sup>-/-</sup> and seven cPLA<sub>2</sub>α<sup>+/-</sup> mice. The incidence of EAE was 100% for cPLA<sub>2</sub>α<sup>+/-</sup> and 0% for cPLA<sub>2</sub>α<sup>-/-</sup> mice. Data are representative of three independent experiments.

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<sub>2</sub>α<sup>+/-</sup> 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<sub>2</sub>α<sup>-/-</sup> mice and spinal cords from three out of six cPLA<sub>2</sub>α<sup>-/-</sup> mice had no infiltrates in any section examined. When present, the changes in cPLA<sub>2</sub>α<sup>-/-</sup> mice were much less severe compared with cPLA<sub>2</sub>α<sup>+/-</sup> mice (Fig. 3). In addition, infiltrates tended to be less common in the parenchyma of the brain and spinal cord



**Figure 3.** Microscopic changes in transverse sections of spinal cords from cPLA<sub>2</sub>α<sup>+/-</sup> and cPLA<sub>2</sub>α<sup>-/-</sup> mice immunized with MOG/CFA and injected with pertussis toxin. Low magnification of thoracolumbar spinal cord from cPLA<sub>2</sub>α<sup>+/-</sup> (A) and cPLA<sub>2</sub>α<sup>-/-</sup> (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<sub>2</sub>α<sup>+/-</sup> mice. There also is vacuolation in the white matter that is consistent with edema. (B) No inflammation in cPLA<sub>2</sub>α<sup>-/-</sup> mouse. Inflammation was absent in 50% of cPLA<sub>2</sub>α<sup>-/-</sup> mice. (C and D). Higher magnification of spinal cords, dorsolateral lumbar region from cPLA<sub>2</sub>α<sup>+/-</sup> (C) and cPLA<sub>2</sub>α<sup>-/-</sup> (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<sub>2</sub>α<sup>+/-</sup> mice. A few dilated axons also are present. (D) When present, inflammation was much less severe in cPLA<sub>2</sub>α<sup>-/-</sup> mice, and was primarily in the leptomeninges.

of cPLA<sub>2</sub>α<sup>-/-</sup> mice compared with cPLA<sub>2</sub>α<sup>+/-</sup> mice. Similar to cPLA<sub>2</sub>α<sup>+/-</sup> mice, the infiltrates in cPLA<sub>2</sub>α<sup>-/-</sup> mice were primarily mononuclear cells.

To determine if cPLA<sub>2</sub>α<sup>-/-</sup> 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<sub>2</sub>α<sup>+/-</sup> and cPLA<sub>2</sub>α<sup>-/-</sup> mice immunized with MOG

Group	Brain		Spinal cord					
	Incidence <sup>a</sup>	Mean no. of foci <sup>b</sup>	Cervical		Thoracic		Lumbar	
			Incidence	Mean no. of foci	Incidence	Mean no. of foci	Incidence	Mean no. of foci
	(%)		(%)		(%)		(%)	
cPLA <sub>2</sub> α <sup>+/-</sup>	6/6 <sup>c</sup> (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 <sub>2</sub> α <sup>-/-</sup>	4/6 (67)	4.8 ± 2.2 <sup>d</sup>	1/6 (17)	4.3 <sup>e</sup> ± 4.3	3/6 (50)	4.0 <sup>d</sup> ± 3.2	3/6 (50)	6.3 <sup>e</sup> ± 2.9

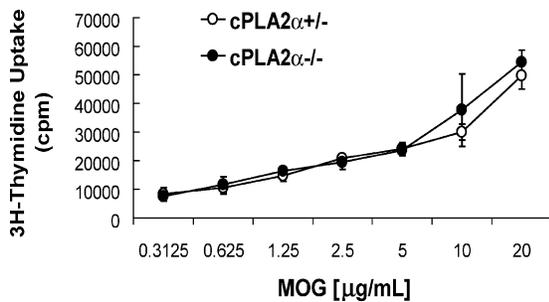
<sup>a</sup>Mice with any inflammatory cell infiltrates were considered positive.

<sup>b</sup>Mean number of foci of ≥20 inflammatory cells ± SEM.

<sup>c</sup>Number of mice affected/number of mice examined (%).

<sup>d</sup>P < 0.0005 compared with cPLA<sub>2</sub>α<sup>+/-</sup>.

<sup>e</sup>P < 0.005 compared with cPLA<sub>2</sub>α<sup>+/-</sup>.



**Figure 4. Proliferation of antigen-specific T cells is not impaired in cPLA<sub>2</sub>α<sup>-/-</sup> mice.** cPLA<sub>2</sub>α<sup>-/-</sup> or cPLA<sub>2</sub>α<sup>+/-</sup> 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 [<sup>3</sup>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.

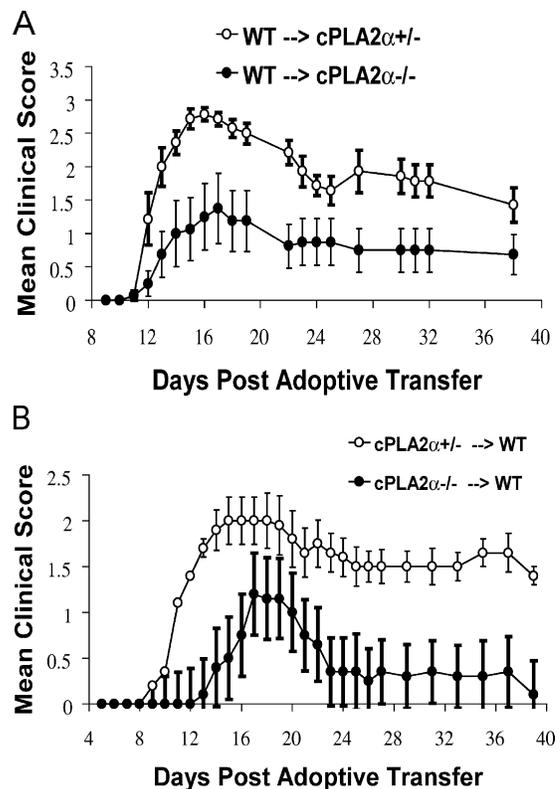
When stimulated with a wide range of concentrations of MOG in vitro, cells that were isolated from the draining LNs of the immunized cPLA<sub>2</sub>α<sup>-/-</sup> and cPLA<sub>2</sub>α<sup>+/-</sup> mice proliferated similarly (Fig. 4). The same results were obtained when purified T cells were isolated from the draining LNs and stimulated with MOG in the presence of WT irradiated B6 spleen cells used as APCs (unpublished data).

#### cPLA<sub>2</sub>α plays important roles in the induction and the effector phases of EAE

After establishing that cPLA<sub>2</sub>α<sup>-/-</sup> mice are resistant to EAE development when immunized with MOG, we sought to determine if cPLA<sub>2</sub>α 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  $5.8 \times 10^6$  cultured spleen cells. We also isolated CD4<sup>+</sup> cells at the end of the culture period; 3 million of these purified CD4<sup>+</sup> 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<sub>2</sub>α 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<sub>2</sub>α<sup>-/-</sup> or cPLA<sub>2</sub>α<sup>+/-</sup> mice. In three independent experiments, cells that were isolated from WT mice in-

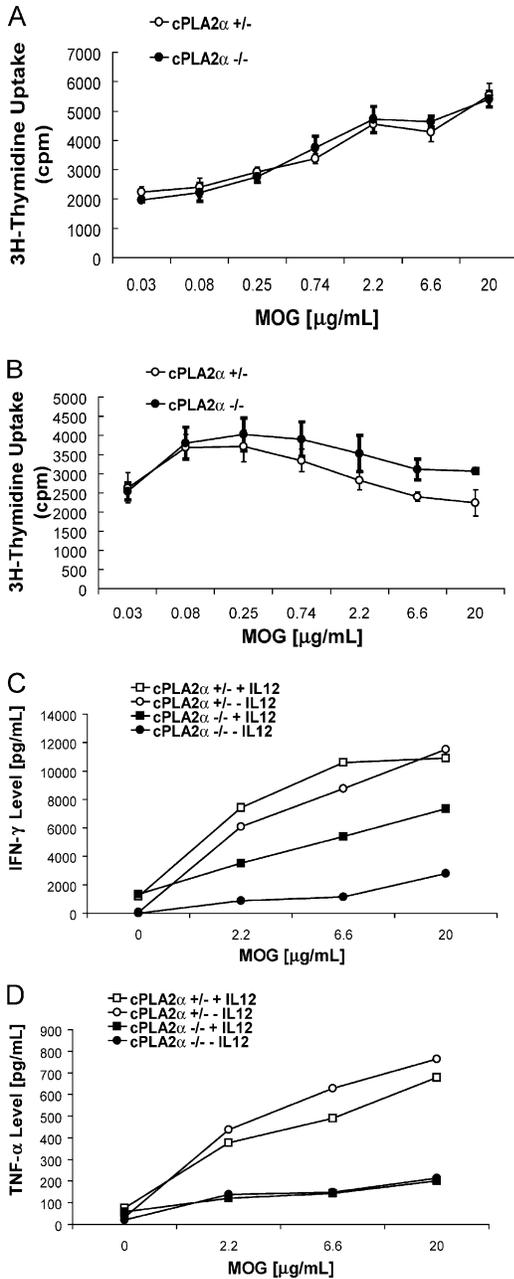


**Figure 5. cPLA<sub>2</sub>α plays a role in the effector and in the induction phases of EAE.** (A) Encephalitogenic cells were prepared by immunizing WT mice and culturing their spleen cells in the presence of MOG and IL-12 for 3 d.  $10^7$  cells were injected i.p. into sublethally irradiated (500 R) cPLA<sub>2</sub>α<sup>-/-</sup> or cPLA<sub>2</sub>α<sup>+/-</sup> mice. EAE clinical scores were assessed as described in Fig 2. Data are shown as a mean clinical score ± SE of eight cPLA<sub>2</sub>α<sup>-/-</sup> mice and seven cPLA<sub>2</sub>α<sup>+/-</sup> mice. The incidence of EAE in the cPLA<sub>2</sub>α<sup>+/-</sup> group was 100% and was 50% in the cPLA<sub>2</sub>α<sup>-/-</sup> 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<sub>2</sub>α<sup>-/-</sup> or cPLA<sub>2</sub>α<sup>+/-</sup> 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<sub>2</sub>α<sup>+/-</sup> cells was 70%; it was 90% in mice that were injected with cPLA<sub>2</sub>α<sup>-/-</sup> 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<sub>2</sub>α<sup>-/-</sup> mice than in cPLA<sub>2</sub>α<sup>+/-</sup> 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<sub>2</sub>α plays a role in the effector phase of EAE. However, because a complete absence of clinical EAE never was observed in cPLA<sub>2</sub>α<sup>-/-</sup> mice that received encephalitogenic cells from WT animals, we tested the possibility that cPLA<sub>2</sub>α also plays a role in the induction of the encephalitogenic immune responses. We immunized cPLA<sub>2</sub>α<sup>-/-</sup> or cPLA<sub>2</sub>α<sup>+/-</sup> 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 orig-

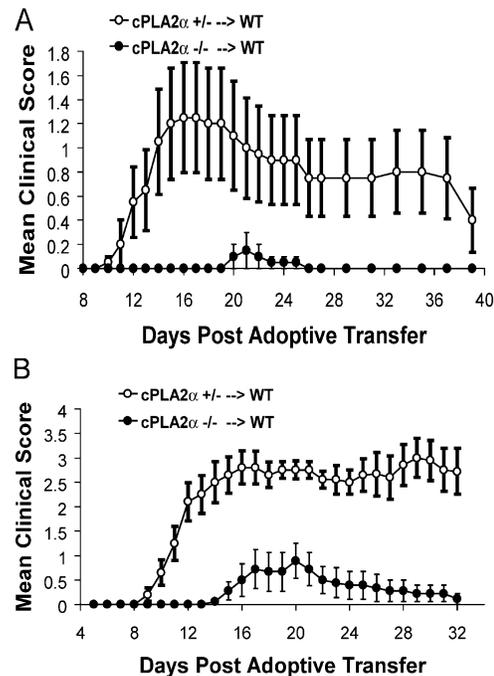
inated from  $cPLA_2\alpha^{-/-}$  mice induced less severe EAE with delayed onset (Fig. 5 B). These results suggest that, in addition to playing a role in the effector phase,  $cPLA_2\alpha$  plays a role in the induction phase of the disease.



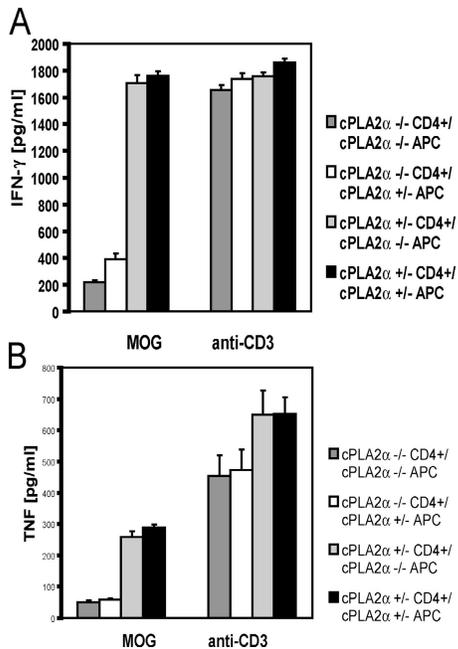
**Figure 6. Proliferation and cytokine production by splenic cells from immunized  $cPLA_2\alpha^{-/-}$  and  $cPLA_2\alpha^{+/-}$  mice stimulated with MOG.** The mice were immunized at two sites s.c. with MOG/CFA, and spleens were collected 10 d later. T cell stimulation was set up in the presence 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- $\gamma$  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



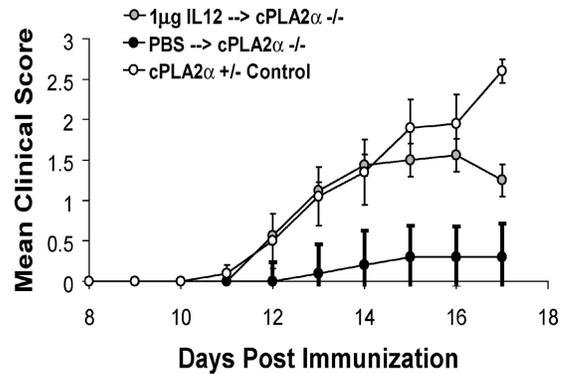
**Figure 7. Absence of IL-12 during the culture period impaired the ability of T cells from  $cPLA_2\alpha^{-/-}$  mice to induce EAE.** Encephalitogenic cells were prepared by immunizing  $cPLA_2\alpha^{-/-}$  or  $cPLA_2\alpha^{+/-}$  mice and culturing their spleen cells in the presence of MOG without addition of exogenous IL-12. In two independent experiments, (A)  $10^7$  or (B)  $4.5 \times 10^7$  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- $\gamma$  or (B) TNF was measured after stimulation with MOG (20  $\mu$ g/ml) or anti-CD3 (1  $\mu$ g/ml).

various cytokines in these MOG-stimulated cultures, a striking reduction in IFN- $\gamma$  levels was observed in cultures of  $cPLA_2\alpha^{-/-}$  cells when compared with  $cPLA_2\alpha^{+/+}$  controls (Fig. 6 C). These reduced IFN- $\gamma$  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 ( $4.5 \times 10^7$ ) to induce EAE,  $cPLA_2\alpha^{+/+}$  cells induced severe disease, whereas cells from



**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  $\pm$  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,  $P = 0.416$ .

$cPLA_2\alpha^{-/-}$  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  $cPLA_2\alpha^{-/-}$  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  $cPLA_2\alpha^{-/-}$  and  $cPLA_2\alpha^{+/+}$  mice. When  $CD4^+$  cells and APC cells were from  $cPLA_2\alpha^{-/-}$  mice, stimulation by MOG produced eight- and fivefold lower levels of IFN- $\gamma$  and TNF, respectively, compared with the levels that were produced in co-cultures of the cells from  $cPLA_2\alpha^{+/+}$  mice (Fig. 8). Much of this difference was maintained when  $CD4^+$  cells from  $cPLA_2\alpha^{-/-}$  and  $cPLA_2\alpha^{+/+}$  mice were stimulated with APCs from the  $cPLA_2\alpha^{+/+}$  mice. However, the source of APCs did have an effect on IFN- $\gamma$  levels when  $CD4^+$  cells from  $cPLA_2\alpha^{-/-}$  mice were stimulated with MOG and APCs from  $cPLA_2\alpha^{+/+}$  mice. The levels of IFN- $\gamma$  produced were almost twice as high as the levels that were produced in cultures in which APCs were from  $cPLA_2\alpha^{-/-}$  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  $cPLA_2\alpha^{+/+}$  mice, the cultures were stimulated with anti-CD3 mAb's. In these cultures, T

cells from  $cPLA_2\alpha^{-/-}$  and  $cPLA_2\alpha^{+/-}$  mice produced similar levels of IFN- $\gamma$ , regardless of the source of the APCs. Amounts of TNF produced by  $cPLA_2\alpha^{-/-}$  CD4 cells were lower ( $\sim 30\%$ ) than amounts of TNF that were produced by CD4 $^+$  cells from  $cPLA_2\alpha^{+/-}$  mice. This indicated that an intrinsic defect in production of TNF may exist in cells from  $cPLA_2\alpha^{-/-}$  mice. However, this defect is much less pronounced than the fivefold defect in TNF that was observed for MOG-specific T cells from  $cPLA_2\alpha^{-/-}$  mice.

#### Injection of IL-12 during disease induction period renders $cPLA_2\alpha^{-/-}$ 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  $\mu\text{g}$  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 AACOCF $_3$ , 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 le-

sions 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  $\sim 50\%$  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<sub>2</sub>α also plays a role in the induction of encephalitogenic immune response.

The reduced encephalitogenic potential of the cPLA<sub>2</sub>α<sup>-/-</sup> 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<sub>2</sub>α<sup>-/-</sup> mice, which indicated that priming and expansion of MOG-specific T cells was not affected by the defect in cPLA<sub>2</sub>α. 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<sub>2</sub>α<sup>-/-</sup> mice. When IL-12 was omitted from the cultures, cells that were isolated from cPLA<sub>2</sub>α<sup>-/-</sup> 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<sup>+</sup> T cells from cPLA<sub>2</sub>α<sup>+/-</sup> mice into cPLA<sub>2</sub>α<sup>-/-</sup> mice does not render cPLA<sub>2</sub>α<sup>-/-</sup> mice susceptible to EAE. Further experiments are required to determine which cells need to produce cPLA<sub>2</sub>α for Th1-type responses to develop. Our results indicate that CD4<sup>+</sup> cells from cPLA<sub>2</sub>α<sup>-/-</sup> 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<sub>2</sub>α-mediated AA release may enhance development of Th1 responses is not known. cPLA<sub>2</sub>α-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<sub>2</sub>α<sup>-/-</sup> 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<sup>-/-</sup> mice at the time of peak disease, which indicated an impaired Th1 response that was analogous to that seen in the cPLA<sub>2</sub>α<sup>-/-</sup> mice (52). Unlike cells that were isolated from cPLA<sub>2</sub>α<sup>-/-</sup> mice, MCP-1<sup>-/-</sup> 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<sub>2</sub>α, MCP-1/CCR2-mediated macrophage trafficking and/or activation is im-

paired, which results in reduced tissue injury and insufficient development or maintenance of Th1 effector cell function.

Products of cPLA<sub>2</sub>α 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>α<sup>-/-</sup> mice have deficient Th1 responses because of reduced IL-23 production. PGE<sub>2</sub> and pharmacologic agonists of the prostaglandin E<sub>2</sub> receptor (EP)2 and EP2/EP4 receptors recently were shown to induce IL-23 production from unstimulated immature DCs (56). cPLA<sub>2</sub>α-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<sub>2</sub>α 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<sub>2</sub>α, induction of Th1-type responses is impaired; we suggest that cPLA<sub>2</sub>α may represent a novel therapeutic target for treatment of MS and other Th1-mediated diseases.

## MATERIALS AND METHODS

**Mice.** cPLA<sub>2</sub>α<sup>-/-</sup> mice on C57BL/6 background were generated by backcrossing cPLA<sub>2</sub>α<sup>-/-</sup> mice of mixed B6/129 background (21) to B6 background for 12 generations and then intercrossing cPLA<sub>2</sub>α<sup>+/-</sup> mice to obtain cPLA<sub>2</sub>α<sup>WT</sup>, cPLA<sub>2</sub>α<sup>+/-</sup>, and cPLA<sub>2</sub>α<sup>-/-</sup> mice, or crossing cPLA<sub>2</sub>α<sup>+/-</sup> and cPLA<sub>2</sub>α<sup>-/-</sup> mice to obtain cPLA<sub>2</sub>α<sup>+/-</sup> and cPLA<sub>2</sub>α<sup>-/-</sup> littermates. All cPLA<sub>2</sub>α 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<sub>2</sub>α<sup>-/-</sup> with cPLA<sub>2</sub>α<sup>+/-</sup> and/or cPLA<sub>2</sub>α<sup>WT</sup> 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<sub>2</sub>α<sup>-/-</sup> 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  $6 \times 10^6$  cells/ml and 20  $\mu\text{g}$  MOG<sub>35-55</sub>/ml in T cell medium (RPMI media enriched with 10% FBS, 2 mM L-glutamine,  $5 \times 10^{-5}$  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<sub>2</sub> 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 lumbar 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<sub>2</sub> and LTB<sub>4</sub> 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  $-80^\circ\text{C}$ . To remove the tissue residues, the extracts were centrifuged at 1,800  $g$  for 20 min at  $4^\circ\text{C}$ . 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<sub>2</sub> and LTB<sub>4</sub> 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<sup>+</sup> and non-CD4<sup>+</sup> (APC) cells were isolated using Dynal beads (Invitrogen), according to the manufacturer's instructions. When CD4<sup>+</sup> and non-CD4<sup>+</sup> cells were isolated, they were mixed at 1:1 ratio.  $8 \times 10^5$  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  $\mu\text{Ci}$  of [<sup>3</sup>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 [<sup>3</sup>H]thymidine incorporation. Concentrations of IL-4, IL-5, IFN- $\gamma$ , 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 non-irradiated, recipient mice. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20050665/DC1>.

Statistics on the microscopic changes were done by Dr. Y. Huang.

T. Shinizu is supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

S. Marusic, M.W. Leach, J.W. Pelker, J. Cui, C.M. DeClercq, M.W.H. Shen, J.S. Miyashiro, B.A. Carito, P. Thakker, D.L. Simmons, and J.D. Clark work for Wyeth and own Wyeth stock and/or Wyeth stock options. Wyeth has a cPLA2 inhibitor currently in clinical trials.

Submitted: 31 March 2005

Accepted: 12 August 2005

#### REFERENCES

- Zamvil, S.S., and L. Steinman. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol.* 8:579–621.
- Martin, R., and H.F. McFarland. 1995. Immunological aspects of experimental allergic encephalomyelitis and multiple sclerosis. *Crit. Rev. Clin. Lab. Sci.* 32:121–182.
- Steinman, L. 1999. Assessment of animal models for MS and demyelinating disease in the design of rational therapy. *Neuron.* 24:511–514.
- Steinman, L., R. Martin, C. Bernard, P. Conlon, and J.R. Oksenberg. 2002. Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. *Annu. Rev. Neurosci.* 25:491–505.
- Kuchroo, V.K., C.A. Martin, J.M. Greer, S.T. Ju, R.A. Sobel, and M.E. Dorf. 1993. Cytokines and adhesion molecules contribute to the ability of myelin proteolipid protein-specific T cell clones to mediate experimental allergic encephalomyelitis. *J. Immunol.* 151:4371–4382.
- Kennedy, M.K., D.S. Torrance, K.S. Picha, and K.M. Mohler. 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J. Immunol.* 149:2496–2505.
- Baron, J.L., J.A. Madri, N.H. Ruddle, G. Hashim, and C.A. Janeway Jr. 1993. Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J. Exp. Med.* 177:57–68.
- Miller, S.D., and W.J. Karpus. 1994. The immunopathogenesis and regulation of T-cell-mediated demyelinating diseases. *Immunol. Today.* 15:356–361.
- Steinman, L. 1996. Multiple sclerosis: a coordinated immunological at-

- tack against myelin in the central nervous system. *Cell*. 85:299–302.
10. Leonard, J.P., K.E. Waldburger, and S.J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med.* 181:381–386.
  11. Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, et al. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*. 421:744–748.
  12. Goldberg, R., Y. Zohar, G. Wildbaum, Y. Geron, G. Maor, and N. Karin. 2004. Suppression of ongoing experimental autoimmune encephalomyelitis by neutralizing the function of the p28 subunit of IL-27. *J. Immunol.* 173:6465–6471.
  13. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3:133–146.
  14. Szabo, S.J., B.M. Sullivan, S.L. Peng, and L.H. Glimcher. 2003. Molecular mechanisms regulating Th1 immune responses. *Annu. Rev. Immunol.* 21:713–758.
  15. Constantinescu, C.S., M. Wysocka, B. Hilliard, E.S. Ventura, E. Lavi, G. Trinchieri, and A. Rostami. 1998. Antibodies against IL-12 prevent superantigen-induced and spontaneous relapses of experimental autoimmune encephalomyelitis. *J. Immunol.* 161:5097–5104.
  16. Becher, B., B.G. Durell, and R.J. Noelle. 2002. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J. Clin. Invest.* 110:493–497.
  17. Dore-Duffy, P., and C. Donovan. 1991. Functional desensitization of monocytes from patients with multiple sclerosis due to prostaglandin E. *Clin. Immunol. Immunopathol.* 61:119–128.
  18. Neu, I.S., G. Metzger, J. Zschocke, R. Zelezny, and E. Mayatepek. 2002. Leukotrienes in patients with clinically active multiple sclerosis. *Acta Neurol. Scand.* 105:63–66.
  19. Callea, L., M. Arese, A. Orlandini, C. Bargnani, A. Priori, and F. Busolino. 1999. Platelet activating factor is elevated in cerebral spinal fluid and plasma of patients with relapsing-remitting multiple sclerosis. *J. Neuroimmunol.* 94:212–221.
  20. Bonventre, J.V., Z. Huang, M.R. Taheri, E. O'Leary, E. Li, M.A. Moskowitz, and A. Sapirstein. 1997. Reduced fertility and postischemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature*. 390:622–625.
  21. Uozumi, N., K. Kume, T. Nagase, N. Nakatani, S. Ishii, F. Tashiro, Y. Komagata, K. Maki, K. Ikuta, Y. Ouchi, et al. 1997. Role of cytosolic phospholipase A2 in allergic response and parturition. *Nature*. 390:618–622.
  22. Kalyvas, A., and S. David. 2004. Cytosolic phospholipase A2 plays a key role in the pathogenesis of multiple sclerosis-like disease. *Neuron*. 41:323–335.
  23. Locati, M., D. Zhou, W. Luini, V. Evangelista, A. Mantovani, and S. Sozzani. 1994. Rapid induction of arachidonic acid release by monocyte chemotactic protein-1 and related chemokines. Role of Ca<sup>2+</sup> influx, synergism with platelet-activating factor and significance for chemotaxis. *J. Biol. Chem.* 269:4746–4753.
  24. Murakami, M., Y. Nakatani, G. Atsumi, K. Inoue, and I. Kudo. 1997. Regulatory functions of phospholipase A2. *Crit. Rev. Immunol.* 17:225–283.
  25. Ransohoff, R.M. 1999. Mechanisms of inflammation in MS tissue: adhesion molecules and chemokines. *J. Neuroimmunol.* 98:57–68.
  26. Kudo, I., and M. Murakami. 2002. Phospholipase A2 enzymes. *Prostaglandins Other Lipid Mediat.* 68–69:3–58.
  27. Funk, C.D. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*. 294:1871–1875.
  28. McMahon, B., and C. Godson. 2004. Lipoxins: endogenous regulators of inflammation. *Am. J. Physiol. Renal Physiol.* 286:F189–F201.
  29. Serhan, C.N. 2005. Novel omega-3-derived local mediators in anti-inflammation and resolution. *Pharmacol. Ther.* 105:7–21.
  30. Huang, J.T., J.S. Welch, M. Ricote, C.J. Binder, T.M. Willson, C. Kelly, J.L. Witztum, C.D. Funk, D. Conrad, and C.K. Glass. 1999. Interleukin-4-dependent production of PPAR- $\gamma$  ligands in macrophages by 12/15-lipoxygenase. *Nature*. 400:378–382.
  31. Niino, M., K. Iwabuchi, S. Kikuchi, M. Ato, T. Morohashi, A. Ogata, K. Tashiro, and K. Onoe. 2001. Amelioration of experimental autoimmune encephalomyelitis in C57BL/6 mice by an agonist of peroxisome proliferator-activated receptor- $\gamma$ . *J. Neuroimmunol.* 116:40–48.
  32. Riendeau, D., J. Guay, P.K. Weech, F. Laliberte, J. Yergey, C. Li, S. Desmarais, H. Perrier, S. Liu, D. Nicoll-Griffith, et al. 1994. Arachidonyl trifluoromethyl ketone, a potent inhibitor of 85-kDa phospholipase A2, blocks production of arachidonate and 12-hydroxyeicosatetraenoic acid by calcium ionophore-challenged platelets. *J. Biol. Chem.* 269:15619–15624.
  33. Ghomashchi, F., R. Loo, J. Balsinde, F. Bartoli, R. Apitz-Castro, J.D. Clark, E.A. Dennis, and M.H. Gelb. 1999. Trifluoromethyl ketones and methyl fluorophosphonates as inhibitors of group IV and VI phospholipases A(2): structure-function studies with vesicle, micelle, and membrane assays. *Biochim. Biophys. Acta.* 1420:45–56.
  34. Koutek, B., G.D. Prestwich, A.C. Howlett, S.A. Chin, D. Salehani, N. Akhavan, and D.G. Deutsch. 1994. Inhibitors of arachidonoyl ethanolamide hydrolysis. *J. Biol. Chem.* 269:22937–22940.
  35. Fowler, C.J., G. Tiger, and A. Stenstrom. 1997. Ibuprofen inhibits rat brain deamidation of anandamide at pharmacologically relevant concentrations. Mode of inhibition and structure-activity relationship. *J. Pharmacol. Exp. Ther.* 283:729–734.
  36. Croxford, J.L., and S.D. Miller. 2003. Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R+WIN55,212. *J. Clin. Invest.* 111:1231–1240.
  37. Amor, S., N. Groome, C. Lington, M.M. Morris, K. Dornmair, M.V. Gardinier, J.M. Matthieu, and D. Baker. 1994. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J. Immunol.* 153:4349–4356.
  38. Lington, C., T. Berger, L. Perry, S. Weerth, D. Hinze-Selch, Y. Zhang, H.C. Lu, H. Lassmann, and H. Wekerle. 1993. T cells specific for the myelin oligodendrocyte glycoprotein mediate an unusual autoimmune inflammatory response in the central nervous system. *Eur. J. Immunol.* 23:1364–1372.
  39. Reese, L., T.J. Carr, R.L. Nicholson, and E.K. Lepp. 1986. Magnetic resonance imaging for detecting lesions of multiple sclerosis: comparison with computed tomography and clinical assessment. *CMAJ.* 135:639–643.
  40. Cross, A.H., B. Cannella, C.F. Brosnan, and C.S. Raine. 1990. Homing to central nervous system vasculature by antigen-specific lymphocytes. I. Localization of 14C-labeled cells during acute, chronic, and relapsing experimental allergic encephalomyelitis. *Lab. Invest.* 63:162–170.
  41. Murphy, P.M. 1994. The molecular biology of leukocyte chemoattractant receptors. *Annu. Rev. Immunol.* 12:593–633.
  42. Tager, A.M., S.K. Bromley, B.D. Medoff, S.A. Islam, S.D. Bercury, E.B. Friedrich, A.C. Carafone, R.E. Gerszten, and A.D. Luster. 2003. Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. *Nat. Immunol.* 4:982–990.
  43. Goodarzi, K., M. Goodarzi, A.M. Tager, A.D. Luster, and U.H. von Andrian. 2003. Leukotriene B4 and BLT1 control cytotoxic effector T cell recruitment to inflamed tissues. *Nat. Immunol.* 4:965–973.
  44. Ott, V.L., J.C. Camabier, J. Kappler, P. Marrack, and B.J. Swanson. 2003. Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4. *Nat. Immunol.* 4:974–981.
  45. Tager, A.M., and A.D. Luster. 2003. BLT1 and BLT2: the leukotriene B(4) receptors. *Prostaglandins Leukot. Essent. Fatty Acids.* 69:123–134.
  46. Park, T.S., E.R. Gonzales, and J.M. Gidday. 1999. Platelet-activating factor mediates ischemia-induced leukocyte-endothelial adherence in newborn pig brain. *J. Cereb. Blood Flow Metab.* 19:417–424.
  47. Kihara, Y., S. Ishii, Y. Kita, A. Toda, A. Shimada, and T. Shimizu. Dual phase regulation of experimental allergic encephalomyelitis by platelet-activating factor. *J. Exp. Med.* 202:853–863.
  48. Locati, M., G. Lamorte, W. Luini, M. Introna, S. Bernasconi, A. Mantovani, and S. Sozzani. 1996. Inhibition of monocyte chemotaxis to C-C chemokines by antisense oligonucleotide for cytosolic phospholipase A2. *J. Biol. Chem.* 271:6010–6016.
  49. Tran, E.H., K. Hoekstra, N. van Rooijen, C.D. Dijkstra, and T. Owens. 1998. Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte

- extravasation from blood, are prevented in macrophage-depleted mice. *J. Immunol.* 161:3767–3775.
50. Reiseter, B.S., G.T. Miller, M.P. Happ, and M.T. Kasaian. 1998. Treatment of murine experimental autoimmune encephalomyelitis with a myelin basic protein peptide analog alters the cellular composition of leukocytes infiltrating the cerebrospinal fluid. *J. Neuroimmunol.* 91:156–170.
  51. Fife, B.T., G.B. Huffnagle, W.A. Kuziel, and W.J. Karpus. 2000. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 192:899–905.
  52. Huang, D.R., J. Wang, P. Kivisakk, B.J. Rollins, and R.M. Ransohoff. 2001. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J. Exp. Med.* 193:713–726.
  53. Emerson, M.R., and S.M. LeVine. 2004. Experimental allergic encephalomyelitis is exacerbated in mice deficient for 12/15-lipoxygenase or 5-lipoxygenase. *Brain Res.* 1021:140–145.
  54. Harizi, H., M. Juzan, V. Pitard, J.F. Moreau, and N. Gualde. 2002. Cyclooxygenase-2-induced prostaglandin e(2) enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J. Immunol.* 168:2255–2263.
  55. Rieser, C., G. Bock, H. Klocker, G. Bartsch, and M. Thurnher. 1997. Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. *J. Exp. Med.* 186:1603–1608.
  56. Sheibanie, A.F., I. Tadmori, H. Jing, E. Vassiliou, and D. Ganea. 2004. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. *FASEB J.* 18:1318–1320.
  57. Hegen, M., L. Sun, N. Uozumi, K. Kume, M.E. Goad, C.L. Nickerson-Nutter, T. Shimizu, and J.D. Clark. 2003. Cytosolic phospholipase A2alpha-deficient mice are resistant to collagen-induced arthritis. *J. Exp. Med.* 197:1297–1302.
  58. Sobel, R.A., V.K. Tuohy, Z.J. Lu, R.A. Laursen, and M.B. Lees. 1990. Acute experimental allergic encephalomyelitis in SJL/J mice induced by a synthetic peptide of myelin proteolipid protein. *J. Neuro-pathol. Exp. Neurol.* 49:468–479.
  59. Sobel, R.A., and V.K. Kuchroo. 1992. The immunopathology of acute experimental allergic encephalomyelitis induced with myelin proteolipid protein. T cell receptors in inflammatory lesions. *J. Immunol.* 149:1444–1451.