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RESEARCH ARTICLE

Plasma phospholipid transfer protein (PLTP) modulates adaptive immune functions through alternation of T helper cell polarization

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Objective: Plasma phospholipid transfer protein (PLTP) is a key determinant of lipoprotein metabolism, and both animal and human studies converge to indicate that PLTP promotes atherogenesis and its thromboembolic complications. Moreover, it has recently been reported that PLTP modulates inflammation and immune responses. Although earlier studies from our group demonstrated that PLTP can modify macrophage activation, the implication of PLTP in the modulation of T-cell-mediated immune responses has never been investigated and was therefore addressed in the present study. Approach and results: In the present study, we demonstrated that PLTP deficiency in mice has a profound effect on $CD4^+$ Th0 cell polarization, with a shift towards the anti-inflammatory Th2 phenotype under both normal and pathological conditions. In a model of contact hypersensitivity, a significantly impaired response to skin sensitization with the hapten-2,4-dinitrofluorobenzene (DNFB) was observed in PLTP-deficient mice compared to wild-type (WT) mice. Interestingly, PLTP deficiency in mice exerted no effect on the counts of total white blood cells, lymphocytes, granulocytes, or monocytes in the peripheral blood. Moreover, PLTP deficiency did not modify the amounts of $CD4^+$ and $CD8^+$ T lymphocyte subsets. However, PLTP-deficiency, associated with upregulation of the Th2 phenotype, was accompanied by a significant decrease in the production of the pro-Th1 cytokine interleukin 18 by accessory cells. Conclusions: For the first time, this work reports a physiological role for PLTP in the polarization of $CD4^+$ T cells toward the pro-inflammatory Th1 phenotype.
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INTRODUCTION

Like plasma cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP) is a member of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) gene family.[1] PLTP is a major regulator of lipoprotein metabolism. Several studies indicate that PLTP promotes atherogenesis and thromboembolic complications.[2,3] Recent studies have clearly shown that PLTP increases the production of apolipoprotein B (apoB)-containing lipoproteins by the liver,[4–6] decreases antioxidants,[7] and increases vascular thrombosis.[8] Recently, genetic variations at the PLTP locus leading to decreased PLTP activity were found to be related to reduced cardiovascular risk, suggesting that PLTP may constitute an emerging cardiovascular risk factor.[9]

Beyond its impact on lipoprotein metabolism, PLTP has recently been reported to modulate inflammation and immune responses. Thus, PLTP deficiency in mice is associated with lower circulating levels of interleukin 6 (IL-6), increased anti-inflammatory properties of high-density lipoproteins (HDL), and the reduced ability of low-density lipoproteins (LDL) to induce monocyte chemotactic activity, thus highlighting the pro-inflammatory effects of PLTP *in vivo*.[10] PLTP in plasma resides on lipid-poor complexes dominated by clusterin and proteins implicated in host defense and inflammation,[11] and a

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positive relationship has been established between PLTP activity and inflammatory markers in patients with cardiovascular disease or diabetes.[12,13] Atherosclerosis is recognized as a chronic inflammatory disease, and both the innate and acquired immune responses have been shown to modulate disease progression.[14–16] While innate immune responses mainly involve monocytes/macrophages, which constitute the hallmark of atherosclerotic lesions, the main component of the adaptive immune response in atherosclerosis is CD4+ T cells.[17,18] Although earlier studies from our group and others have demonstrated that PLTP can modify macrophagefunctions,[19,20] the role of PLTP in the modulation of CD4+ T-cell-mediated acquired immune responses has never been investigated.

Using the PLTP-deficient mouse model, we demonstrated a profound effect of PLTP onCD4+ T-cell polarization towards the pro-inflammatory Th1 subtype. This effect may constitute an additional feature of the proatherogenic potency of PLTP.

MATERIALS AND METHODS

Animals

Four- to six-month-old PLTP-deficient homozygous ($PLTP^{-/-}$) and WT mice on a homogeneous C57BL/6 background were used in the present study. PLTP-deficient mice were generated and kindly provided by Drs. X.C. Jiang and A.R. Tall (Columbia University, New York). The animals (4–6 months old) had free access to water and food and were fed a standard chow diet (A03 diet, Safe, Augy, France). All experiments involving animals were conducted in accordance with institutional guidelines and were approved by the University of Burgundy's Ethics Committee on the Use of Laboratory Animals (protocol number 3103).

Blood sampling

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine, and blood was collected via cardiac puncture into syringes containing sodium citrate (1:10, v/v) (Sigma, Saint Quentin Fallavier, France). Plasma was obtained by centrifugation (15 min, 3000× *g*, 4 °C), and the plasma was stored at −80 °C until analysis.

T-cell type and quantity

The percentages of $CD3^+$, $CD4^+$, $CD8^+$, and $CD25^+$ T cells were determined among total splenocytes of $PLTP^{-/-}$ and WT mice by using the mouse T lymphocyte subset antibody cocktail (BD Biosciences, Erembodegem, Belgium) and a rat anti-mouse CD25-FITC antibody (BD Biosciences) on a LSRII flow cytometer (BD Biosciences).

Culture of spleen mononuclear cells and T-cell activation

Each spleen was placed in RPMI 1640 culture medium (Invitrogen/Thermo Fisher Scientific, Illkirch, France). The spleens were passed through a sterile wire mesh to remove any tissue debris. After centrifugation, the pellets were incubated with 0.83% NH_4Cl Tris buffer to lyse the red blood cells. The splenocytes were washed and resuspended in culture medium. The mononuclear cells were isolated by centrifugation on a 4% Ficoll gradient, washed twice, and plated in 24-well plates at 2.10^6 cells mL^{-1} in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After 2 h, the T lymphocytes were activated by the addition of 2 μg mL^{-1} anti-CD3 (Serotec/Bio-Rad, Kidlington, UK) and 5 μg mL^{-1} anti-CD28 (BD Biosciences) antibodies. The culture media were harvested after 36 h and centrifuged. The pellets were resuspended in TRIzol reagent and stored at −20 °C until RNA extraction, and the supernatants were stored at −80 °C.

RNA extraction and RT-PCR

Total RNA was isolated from axillary lymph nodes, spleen mononuclear cells, and $CD4^+CD25^+$ regulatory T cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The Tbet, GATA3, IL-10, CTLA-4, and FoxP3 mRNA levels were quantified by reverse transcription followed by real-time PCR using a Light Cycler detection system (Roche, Basel, Switzerland). Amplification was undertaken using a Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) according to the instructions provided by the manufacturer. GAPDH was used as a reference gene, and the results were expressed as the ratio between the RNA expression level of the target and reference genes.

Oligonucleotide primers used for cDNA amplification were as follows:

GAPDH, sense: TGCCATTTGCAGTGGCAA;
antisense: TTCCAGAGGGGCCATCCA
Tbet, sense: GCCAGGGAACCGCTTATATG;
antisense: GACGATCATCTGGGTCACATTGT
GATA3, sense: TCGGCCATTCGTACATGGAA;
antisense: GTCGGAGGATACCTCTGCA
FoxP3, sense: CCTATGGCTCCTTCCTTGGC;
antisense: ATGAAGTGTGGTCTGTCCTGG
CTLA-4, sense: TACTTTGTGGGCATGGGCAA;
antisense: AGAAACAGCAGTGACCAGGAA
IL-10, sense: GGTGTCCTTTCAATTGCTCTCA;
antisense: ACAGTAGGGGAACCCTCTGA

Assay of Th1/Th2 cytokines, IL17A, and TGF-β

Interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 10 (IL-10), interferon gamma (IFN-γ), IL-17A and transforming growth factor beta 1 (TGF-β1) were assayed in plasma or culture supernatants using commercial enzyme-linked immunosorbent assay (ELISA) kits (Mouse Th1/Th2 and IL17A Ready-SET-Go! ELISA Sets, Human/Mouse TGF-β1 ELISA Ready-Set-Go!, eBiosciences, San Diego, USA).

Contact hypersensitivity protocol

The abdominal skin of the mice was shaved and sensitized with DNFB (Sigma) (0.5%) in acetone/olive oil (4:1).[1] Five days later, the dorsal surface of the right ear was challenged with 0.2% DNFB. Acetone/olive oil was applied to the left ear to serve as a control. Ear thickness was measured in four points before challenge and 48 h after challenge using a micrometer (Labo Ax), and ear swelling was calculated as the difference between right (sensitized) and left (non-sensitized) ear thickness at a specific point. The ears were harvested and embedded

in paraffin for histological analysis, and axillary lymph nodes were harvested for gene expression analysis.

T lymphocyte proliferation test

$CD4^+CD25^-$ and $CD4^+CD25^+$ T lymphocytes were purified from the spleen using MACS Technology ($CD4^+CD25^+$ Regulatory T-cell isolation kit, Miltenyi Biotec, Paris, France). The immunosuppressive activity of regulatory $CD4^+CD25^+$ T cells was assessed using a lymphocyte proliferation test. Briefly, $CD4^+CD25^-$ T cells were plated at 50 000 cells $well^{-1}$ in 96-well culture microplates. The cells were incubated for 40 h at 37 °C in RPMI1640 containing 10% FBS, 1% penicillin/streptomycin, anti-CD3 antibodies (0.1 μg mL^{-1}) and increasing amounts of $CD4^+CD25^+$ regulatory T cells. After a further 6 h of incubation in the presence of ^{3}H-thymidine (0.8 μCi μL^{-1}), $CD4^+CD25^-$ cells were washed and scrapped from the plate, and radioactivity was measured using a liquid scintillation counter (CatherBeckman Coulter, Paris, France).

Statistical analysis

Results are expressed as the mean ± SEM. The statistical significance of differences between the data mean values was determined with the non-parametric Mann–Whitney *U* test.

RESULTS

The effects of PLTP deficiency on Th ($CD3^+CD4^+$), Tc ($CD3^+CD8^+$), and T-Reg ($CD3^+CD4^+CD25^+$) cells

Peripheral white blood cells (WBC) were quantitated in WT and $PLTP^{-/-}$ mice after methylene blue staining of citrated blood samples, and the relative amounts of WBC subpopulations were determined by manual counting. As shown in Table 1, total WBC counts were similar in WT and $PLTP^{-/-}$ mice, as were the relative amounts of lymphocytes, monocytes, and granulocytes.

The relative amounts of helper ($CD3^+CD4^+$), cytotoxic ($CD3^+CD8^+$), and regulatory ($CD4^+CD25^+$) T cells were determined by flow cytometry among total spleen mononuclear cells isolated from WT and $PLTP^{-/-}$ mice (Figure 1a). As shown in Figure 1b, $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD4^+CD25^+$ cells represented approximately 15%, 5%, and 0.5% of total mononuclear splenic cells, respectively. The relative abundance of these cell types was similar in $PLTP^{-/-}$ and WT mice.

Table 1 Blood leukocyte counts in wild-type and $PLTP^{-/-}$ mice. Blood samples were drawn from WT ($n = 6$) and $PLTP^{-/-}$ ($n = 8$) mice, and the leukocyte counts were performed as described in the Materials and Methods section. The results are expressed as the mean ± S.E.M

	Wild-type	$PLTP^{-/-}$
Total leukocytes (cells/mm^3)	5826 ± 612	6373 ± 669
Leukocyte subpopulations (%)		
Lymphocytes	78.0 ± 3.7	78.9 ± 2.0
Eosinophils	1.0 ± 0.4	0.6 ± 0.5
Neutrophils	18.3 ± 3.5	17.9 ± 1.8
Monocytes	2.7 ± 0.9	2.6 ± 0.5

The impact of PLTP deficiency on Th1, Th2, and Th17 cytokine levels

In the first attempt to determine whether PLTP deficiency alters helper T-cell polarization under basal conditions, we measured circulating cytokine levels in the plasma of $PLTP^{-/-}$ and WT mice using ELISA tests. As shown in Figure 2a, the plasma levels of Th2 cytokines (IL-4 and IL-10) were significantly higher in the plasma of $PLTP^{-/-}$ mice than in WT plasma(IL-4: 3.98 ± 0.40 pg mL^{-1} in $PLTP^{-/-}$ versus 2.71 ± 0.42 pg mL^{-1} in WT mice, $p < 0.05$; IL-10: 555.32 ± 62.83 pg mL^{-1} in $PLTP^{-/-}$ versus 317.50 ± 59.74 pg mL^{-1} in WT mice, $p < 0.05$), while concomitant levels of the Th1 cytokine IL-2 were significantly lower (IL-2: 1.13 ± 0.53 pg mL^{-1} in $PLTP^{-/-}$ versus 2.78 ± 0.34 pg mL^{-1} in WT mice, $p < 0.05$). The levels of IFN-γ also tended to be lower, but the difference was not significant (IFN-γ: 24.71 ± 8.67 in $PLTP^{-/-}$ versus 29.43 ± 10.20 pg mL^{-1} in WT mice, n.s.).

When spleen mononuclear cells were isolated and subjected to *in vitro* activation in the presence of anti-CD3 and anti-CD28 antibodies (Figure 2b), we again measured higher production of IL-4 and IL-10 (+158% and +72%, respectively, $p < 0.005$) in $PLTP^{-/-}$ cells than in WT cells, with a concomitant lower production of IL-2 and IFN-γ (−27% and −29%, respectively, $p < 0.02$ for IFN-γ). The level of IL-17A, i.e., the main cytokine product of Th17 cells, was identical in $PLTP^{-/-}$ and WT mice (data not shown). To confirm our results, the expression of the Th1 differentiation marker Tbet and of the Th2 differentiation marker GATA3 were measured by real-time PCR in isolated spleen cells from WT and $PLTP^{-/-}$ mice. As shown in Figure 3, Tbet expression was decreased by almost 4-fold in $PLTP^{-/-}$ splenocytes compared to WT cells, and a concomitant, although not statistically significant, increase in GATA3 expression was observed in $PLTP^{-/-}$ cells. These data confirmed that the ratio of Th1/Th2 lymphocytes was shifted toward a Th2 dominance in $PLTP^{-/-}$ mice.

Taken together, these results indicate that under basal conditions, PLTP deficiency is associated with a significant trend toward Th2 polarization of both circulating and splenic helper T cells.

Delayed-type contact hypersensitivity is compromised in PLTP-deficient mice

To determine the impact of PLTP deficiency on a prototypical Th1-mediated immune response (i.e., contact hypersensitivity), WT and $PLTP^{-/-}$ mice were sensitized by abdominal DNFB painting and challenged 5 days later by an epicutaneous application of DNFB on the right ear. Although both WT and $PLTP^{-/-}$ mice developed ear swelling after DNFB application, as measured by an increase in ear thickness, the ear swelling in $PLTP^{-/-}$ mice was significantly attenuated compared with that measured in WT mice (Figure 4a). The histological analyses showed that the DNFB challenge led to massive edema in the ears of sensitized WT mice. These changes were less prominent

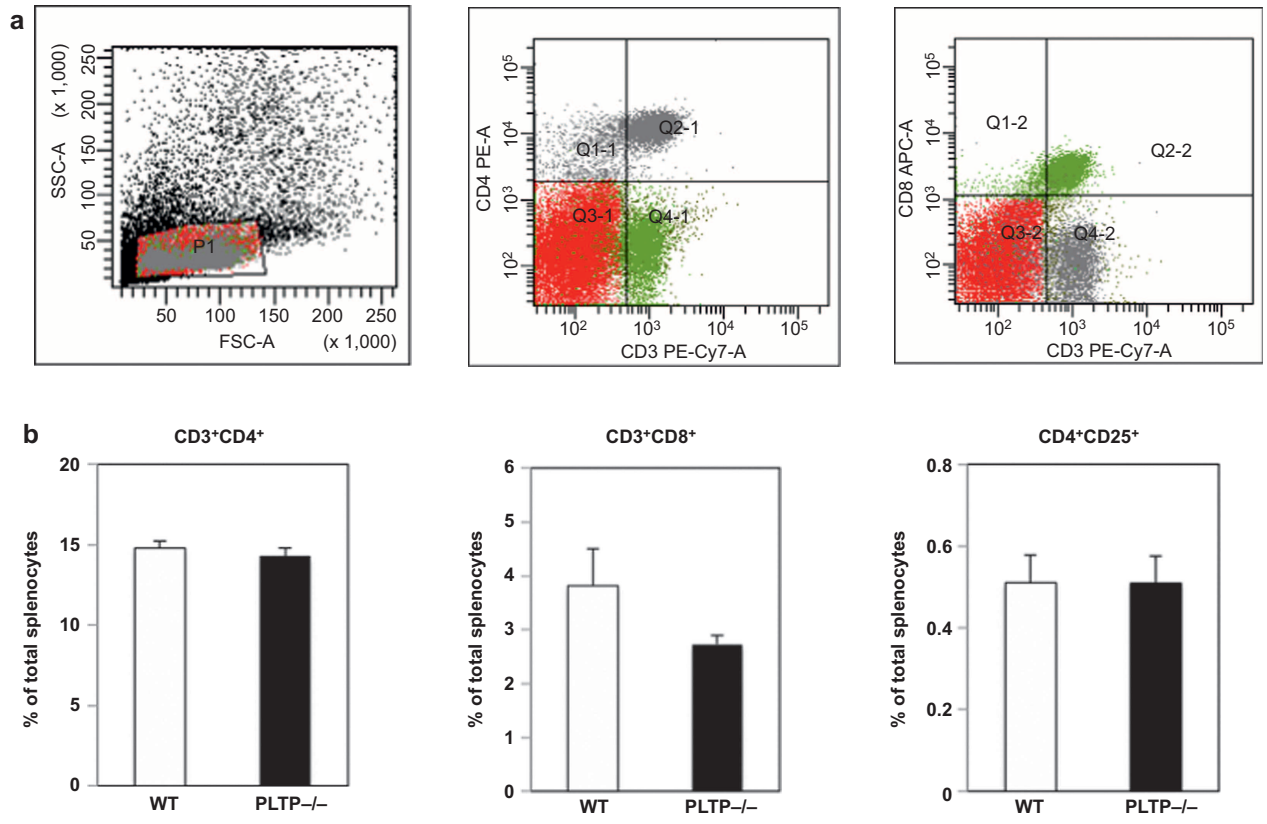

Figure 1 Flow cytometric assessment of helper ($CD4^+$) and cytotoxic ($CD8^+$) T lymphocyte abundance in the spleen of WT and $PLTP^{-/-}$ mice. $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were quantitated by flow cytometry from total splenic mononuclear cells from WT ($n = 11$) and $PLTP^{-/-}$ ($n = 11$) mice, as described in the Materials and Methods section. (**a**) Dot-plots showing the selected cell population (left panel, P1) and the quartiles defined for quantitation of $CD3^+CD4^+$ (middle panel) and $CD3^+CD8^+$ (right panel) cell populations. (**b**) Quantitative analysis (mean ± SEM for each group).

in the ears of sensitized $PLTP^{-/-}$ animals (Figure 4b). The expression level of the T-cell differentiation markers Tbet and GATA3 was determined by real-time PCR in the draining lymph nodes of DNFB-challenged mice. As shown in Figure 4c, expression of the Th1-specific transcription factor Tbet was decreased by approximately 2-fold, whereas that of the Th2-specific transcription factor GATA3 was increased by 2-fold in $PLTP^{-/-}$ compared to WT mice ($p < 0.02$) following DNFB challenge. Thus, PLTP deficiency in mice is associated with an impaired Th1-mediated response to delayed-type contact hypersensitivity.

PLTP deficiency and regulatory T-cell activity

As shown in Figure 1b, the relative proportion of $CD4^+CD25^+$ T cells among total splenocytes was identical in WT and $PLTP^{-/-}$ mice. To assess regulatory T-cell activity, we analyzed the expression of genes implicated in Treg development and function, such as the transcription factor Foxp3, CTLA-4, and IL-10 in $CD4^+CD25^+$ T cells isolated from spleen mononuclear cells of WT and $PLTP^{-/-}$ mice. The purity of the $CD4^+CD25^+$ T cells isolated by magnetic cell sorting was assessed by flow cytometric analysis and found to be greater than 90% (data not shown). The mRNA levels in freshly isolated cells were quantified by real-time PCR. As shown in Figure 5a–c, we found that the mRNA expression levels of Foxp3, CTLA4, and IL-10 were similar in WT and $PLTP^{-/-}$ $CD4^+CD25^+$ T cells. Moreover, the circulating levels of TGF-β, an immunoregulatory cytokine produced by Th3 regulatory cells, were not significantly different in WT and $PLTP^{-/-}$ mice (data not shown). To further assess the immunosuppressive properties of $CD4^+CD25^+$ Treg cells, we used a $CD4^+$ T lymphocyte proliferation test. As shown in Figure 5d, the proliferative capacities of $CD4^+$ cells isolated from either WT or $PLTP^{-/-}$ splenocytes were similar in the absence of regulatory T cells, and the proliferative capacity decreased in a dose-dependent manner in the presence of Treg cells. We found no difference between the immunosuppressive activity of $CD4^+CD25^+$ T cells isolated from WT or $PLTP^{-/-}$ splenocytes, whether they were incubated in the presence of target WT or $PLTP^{-/-}$ $CD4^+$ T cells. Taken together, these results indicate that PLTP deficiency had no impact on the functionality of regulatory T cells.

The effect of PLTP deficiency on antigen-presenting cell-derived products

Because APC-derived products play a major role in T-cell priming and differentiation, we assessed the level of prostaglandin E2, interleukin 12 (IL-12), and interleukin 18

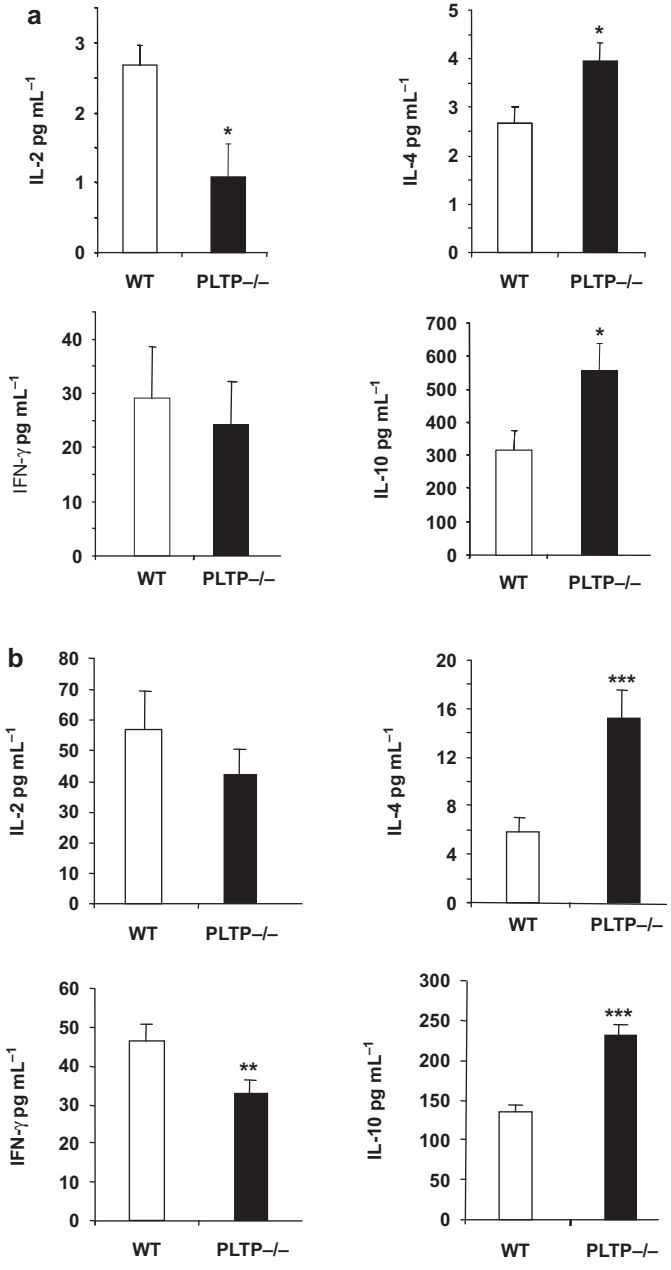

Figure 2 Th1 and Th2 cytokine levels in plasma and splenocyte culture supernatants from PLTP$^{-/-}$ and WT mice. Th1 and Th2 cytokines were measured by ELISA in either plasma samples (panel A) or supernatants of splenocyte cultures after activation with anti-CD3 and anti-CD28 antibodies (panel B). The results are expressed as the mean ± SEM of $n = 6$ WT and $n = 6$ PLTP$^{-/-}$ mice. *$p < 0.05$, **$p < 0.02$, ***$p < 0.005$ versus WT mice (Mann–Whitney U test).

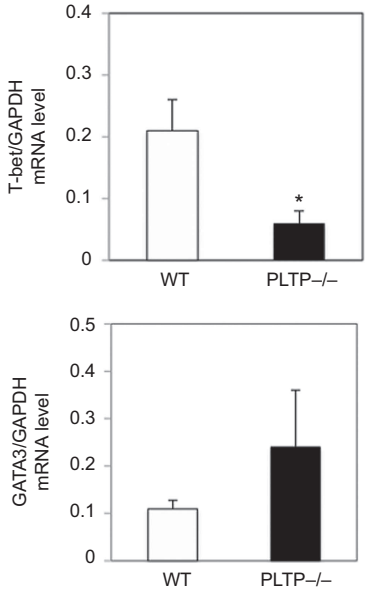

Figure 3 Expression of the Th1 and Th2 differentiation markers Tbet and GATA3 in the splenocytes of WT and PLTP$^{-/-}$ mice. The mRNA expression levels of Th1 and Th2 differentiation markers were determined in spleen mononuclear cells of WT ($n = 4$) and PLTP$^{-/-}$ ($n = 4$) mice by real-time PCR using GAPDH as a reference gene. The results are expressed as the ratio of the mRNA expression level of the target and reference genes (mean ± SEM). *$p < 0.05$ versus WT (Mann Whitney U test).

(IL-18) in the plasma of PLTP$^{-/-}$ and WT mice. As shown in Figure 6, the concentrations of both PGE2 and the pro-Th1 cytokine IL-18were decreased in the plasma of PLTP-deficient mice compared with WT mice(-24.3%, $p < 0.05$ and -46%, $p < 0.01$, respectively). No difference in IL-12 levels was observed between the two genotypes. IL-18 expression was markedly lower (-42%, $p < 0.02$) in isolated PLTP-deficient monocytes than in WT monocytes.

DISCUSSION

We observed that the lack of active circulating PLTP is associated with a shift of Th0 cells toward the anti-inflammatory Th2 phenotype, suggesting that PLTP is implicated in the modulation of the adaptive immune response *in vivo*.

The function and regulation of helper T-cell subsets (Th1, Th2, Th17) are critical to both normal physiological host responses and pathophysiological responses. While Th1 cells are mainly responsible for phagocyte-mediated host defenses and constitute the main effectors of cell-mediated immunity, delayed-type hypersensitivity reactions, and chronic inflammation, Th2 cells promote humoral immunity, allergic reactions and the recruitment of eosinophils.[21] In PLTP$^{-/-}$ animals, an impaired Th1 response and a concomitant increase in the Th2 response were observed in isolated spleen mononuclear cells. *In vivo*, the relevance of these observations was ascertained through (i) the measurement of the plasma levels of Th1 and Th2 cytokines and (ii) the assessment of ear swelling and T-cell orientation in a delayed-type contact hypersensitivity model. It is noteworthy that the number and activity of regulatory T cells and Th17 cells were not altered in PLTP$^{-/-}$ mice compared to WT mice. This observation indicates the following: first, there is a specific impact of PLTP on Th1/Th2 polarization that does not extend to other CD4$^+$ T cells; second, alterations in Treg and/or Th17 cell activity cannot explain the observed alteration of the Th1/Th2 balance.

Our results extend previous findings from our group and others showing that PLTP might play a role in innate immunity and inflammation. Thus, earlier animal studies indicated that

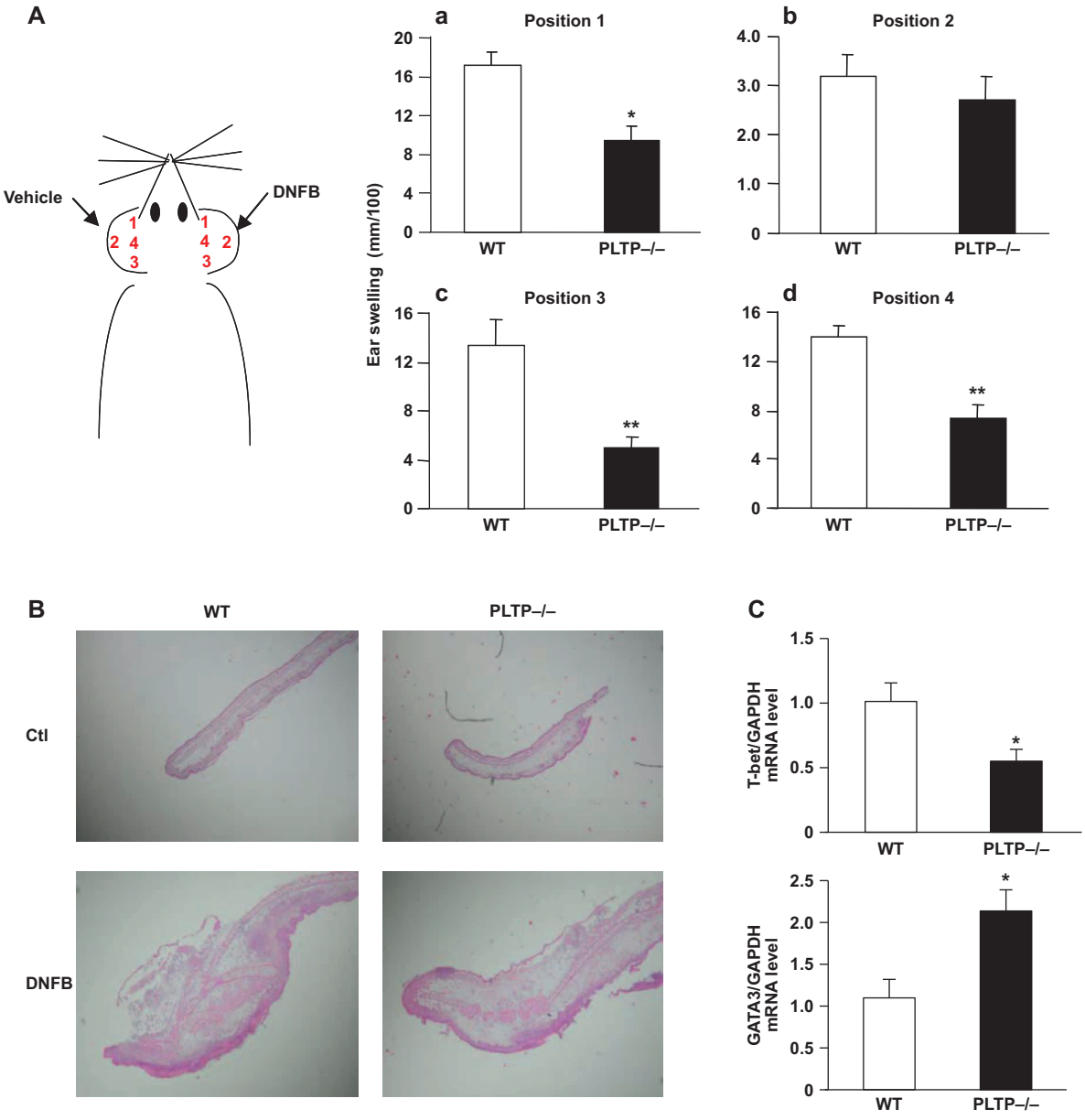

Figure 4 Delayed-type contact hypersensitivity response in WT and PLTP$^{-/-}$ mice. The abdominal skin of the mice was shaved and sensitized with DNFB (Sigma) (0.5%) in acetone/olive oil (4:1). Five days later, the dorsal surface of the right ear was challenged with 0.2% DNFB, and acetone/olive oil was applied to the left ear to serve as a control. The ear thickness was measured at four points before challenge and 48 h after challenge, and ear swelling was calculated as the difference between the ear thicknesses of the sensitized and non-sensitized ears at a specific position (1, 2, 3, or 4). *Panel A*: Schematic representation of measurement points and ear swelling in 1, 2, 3, and 4. *$p < 0.01$, **$p < 0.005$ versus WT (Mann Whitney *U* test). *Panel B*: Histology of non-sensitized (Vehicle) and sensitized (DNFB) ears. The transverse sections of the ear from WT and PLTP$^{-/-}$ mice 48 h after the challenge were stained with hematoxylin and eosin. *Panel C*: Expression of Tbet and GATA3 differentiation markers in axillary lymph nodes of WT and PLTP$^{-/-}$ mice 48 h after the DNFB challenge. *$p < 0.02$ versus WT (Mann Whitney *U* test). The data are presented as the mean ± SEM obtained with a total of $n = 5$ WT and $n = 5$ PLTP$^{-/-}$ mice in two separate experiments.

PLTP deficiency constitutes an anti-inflammatory condition because it is associated with the increased anti-inflammatory properties of HDL and the decreased ability of LDL to induce monocyte chemotactic activity.[10] Moreover, circulating levels of interleukin 6 (IL-6) in PLTP-deficient mice were significantly lower than those in WT mice, and exogenous IL-6 treatment significantly increases the amount of I-a(b)-positive peripheral monocytes in WT but not in PLTP-deficient mice.[22,23] Finally, in a recent study from our group, the expression level of IL-6 and the number of infiltrating macrophages in aortic tissue after the induction of experimental aneurysm were lower in PLTP-deficient mice than in WT mice, providing additional support for the anti-inflammatory effect of PLTP deficiency in mice.[24] With regard to human studies, Cheung

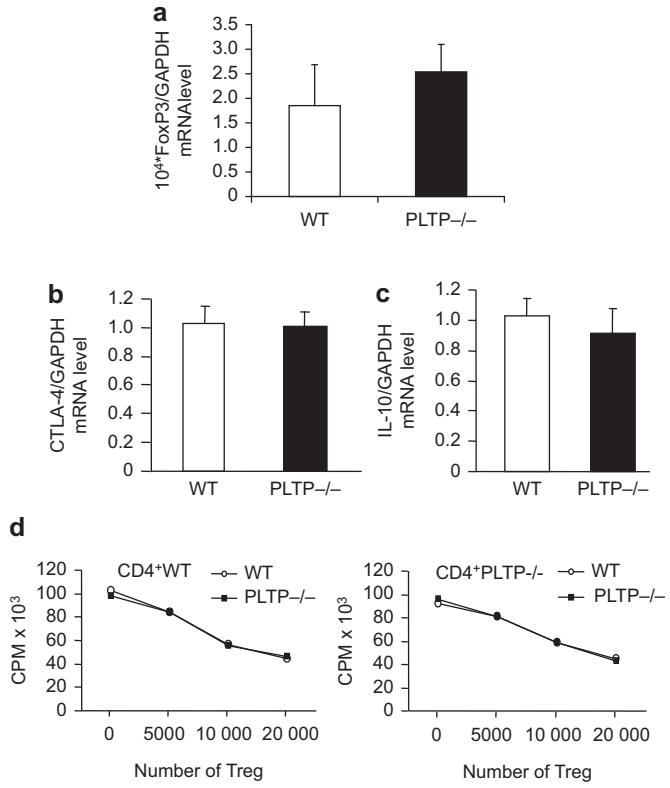

Figure 5 Treg numbers and cell function in WT and PLTP$^{-/-}$ mice. **a–c**: The mRNA expression levels of FoxP3, CTLA4, and IL-10 were quantified in CD4$^+$CD25$^+$ cells isolated from splenic mononuclear cells of WT ($n = 4$) and PLTP$^{-/-}$ ($n = 4$) mice by real-time PCR. GAPDH was used as a reference gene. The results are expressed as the ratio of the mRNA expression level of the target and reference genes (mean ± SEM). D: CD4$^+$CD25$^-$ T (Teff) cells as responder cells and CD4$^+$CD25$^+$ regulatory T (Treg) cells, both purified from the spleens of mice, were co-cultured with soluble anti-CD3 antibodies for 40 h. After a further 6-h incubation in the presence of [^{3}H]thymidine, the cells were washed, and proliferation was measured. The results are representative of three distinct experiments, and each point is the mean ± SEM of four determinations. *Left panel*: WT Teff cells co-incubated with WT (open circles) or PLTP$^{-/-}$ (closed squares) Treg cells. *Right panel*: PLTP$^{-/-}$ Teff cells co-incubated with WT (open circles) or PLTP$^{-/-}$ (closed squares) Treg cells.

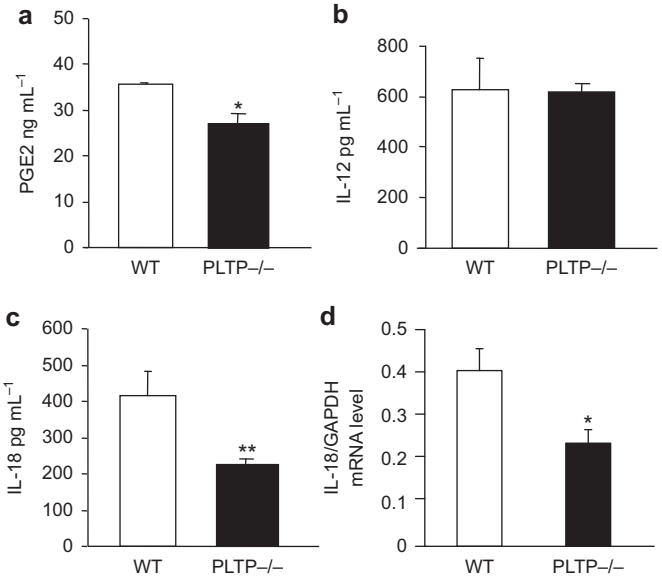

Figure 6 Accessory cell-derived products in WT and PLTP$^{-/-}$ mice. **a–c**: Prostaglandin E2, interleukin 12, and interleukin 18 were quantified in the plasma of WT ($n = 5$) and PLTP$^{-/-}$ ($n = 5$) mice by ELISA. **d**: The expression level of interleukin 18 was measured in accessory cells isolated from the spleens of WT ($n = 6$) and PLTP$^{-/-}$ ($n = 6$) mice. The data are represented as the mean ± SEM. *$p < 0.02$, **$p < 0.01$ versus WT (Mann Whitney *U* test).

et al. recently reported that the majority of PLTP-associated proteins are involved in innate immunity and/or the inflammatory response in plasma,[11] and PLTP activity was found to be significantly increased in patients with bacterial infections and systemic inflammation.[25] In the present work, we observed that neither the total WBC counts nor the CD3$^+$-cell subpopulations were modified in a PLTP-deficient context. This suggests that PLTP does not induce modifications in leukocyte generation or egress from secondary lymphoid tissues; rather, it modulates the balance between Th1 and Th2 subtypes.

The latter effect might be driven by the alteration of intrinsic cell signaling pathways. However, in the present work, no difference in T-cell polarization was revealed when isolated spleen CD4$^+$ T cells, instead of total spleen mononuclear cells, were activated in the presence of anti-CD3 and anti-CD28 antibodies (data not shown). These observations indicate that the impact of PLTP deficiency on the Th1/Th2 balance is unlikely to relate to alterations of the intrinsic properties of CD4$^+$ T cells, but rather, results from modifications of the cytokine milieu defined by accessory cell secretions.

Actually, most of the mechanisms driving the differentiation of naïve CD4$^+$ T cells into Th1 or Th2 phenotypes are linked to the conditions during initial or repeated encounters with the allergen via an antigen-presenting cell (APC). The different types of APCs and their ability to display particular cytokine production profiles, pattern recognition receptors, costimulatory molecules, and specific HLA haplotypes are key determinants of Th1 and Th2 cell polarization. In particular, the ability of APCs to induce Th1 differentiation has been related to the production of high levels of IL-12 and/or IL-18.[26,27] In the present study, we found no difference in IL-12 production by isolated accessory cells from WT and PLTP$^{-/-}$ animals. In sharp contrast, the production of IL-18 (i.e., another potent pro-Th1 cytokine) was markedly reduced in PLTP$^{-/-}$ mice. IL-18 promotes Th1 immune responses through its well-recognized capacity to evoke IFN-γ expression in lymphocytes[27] as well as through IFN-γ-independent pathways, in synergy with IL-12.[28] Our results suggest that the alteration of IL-18 expression by APCs may account at least in part for the switch in the Th1/Th2 balance in PLTP-deficient mice.

The role of PLTP in the polarization of CD4$^+$ T cells toward the pro-inflammatory Th1 phenotype might well constitute one additional feature of its proatherogenic potency. Recent *in vivo* studies in mice and rabbits have led to the identification

of plasma PLTP as a potent pro-atherogenic factor,[9,29] and PLTP deficiency provides protection against atherosclerosis in apoE0 and apoBTg/CETPTg mice, due in part to the decreased production of apoB by the liver and the increased antioxidant protection of atherogenic lipoproteins.[4,7] Interestingly, PLTP deficiency also conferred protection in LDLR0 mice, even though apoB levels were not decreased in this genetic background, suggesting that PLTP deficiency has other anti-atherogenic properties. Atherosclerosis is a multifactorial disease, and the classical view that the development of atherosclerotic lesions solely depends on lipid deposition has now been challenged by more recent studies showing that the activation of inflammatory and immune responses (both innate and adaptive) plays a central role in plaque initiation and progression.[30] The majority of T cells in mouse and human atherosclerotic plaque are $CD4^+$ T cells, and it has been well established that the Th1/Th2 balance can determine the evolution and complications of the plaques.[31] While a series of animal studies have helped establish that Th1 responses have a potent pro-atherogenic effect, there is currently no clear evidence that Th2 responses have an anti-atherogenic effect, and it is currently accepted that a Th1/Th2 switch is protective by alleviating the proatherogenic effects of Th1.[32] As mentioned above, APCs are instrumental in defining the type of effector T cell formed, and both IL-12 and IL-18 are central in Th1 differentiation. The significant decreases in circulating levels of IL-18 and its expression by isolated monocytes that was observed in PLTP-deficient mice may well contribute to the pro-atherogenic potency of PLTP because numerous studies conducted in animal models showed that IL-18 has a pro-atherogenic effect. The overexpression of IL-18 by the injection of an adenoviral vector in ApoE-deficient mice caused an increase in the size of the lesions and the vulnerability of the plaque by the induction of an unstable phenotype.[33,34] In contrast, the deletion of the IL-18 gene in apoE-deficient mice led to reductions in both the atherosclerotic plaques and the levels of Th1 cytokines,[35] and overexpression of IL-18BP, i.e., the endogenous inhibitor of IL-18, reduces the development of atherosclerotic lesions in ApoE-deficient mice and leads to a stable plaque phenotype.[36] Similarly, mice deficient in caspase-1, i.e., the enzyme necessary to convert pro-IL18 into its active form, show increased survival and decreased left ventricular dilatation following myocardial infarction.[37] In humans, IL-18 and its receptors appear to be very highly expressed in all cell types of atherosclerotic plaques[37] and are located preferentially in unstable symptomatic plaques.[38] It is worthy of note that the implication of IL-18 and its receptor in atherogenesis seems to extend beyond the regulation of Th1-mediated immune responses because it promotes the production of IFN-γ by smooth muscle cells in atheroma as well as the expression of adhesion molecules, matrix degrading enzymes, growth factors and cyclooxygenases. They thus promote processes associated with weakening and rupture of the plaques.[36,38] In humans, epidemiological data from case/control studies showed that elevated blood IL-18 levels are associated with an increased risk of acute coronarysyndrome,[39] restenosis,[40] cardiac arrest,[41,42] and myocardial infarction.[43] The serum concentration of IL-18 was connected to cardiovascular mortality during the prospective follow-up of patients in a cohort which included 10 600 healthy European men.[44]

Because PLTP can transfer a variety of amphipathic lipids, including phospholipids, cholesterol, diacylglycerides, and vitamin E, variations in the membrane lipid composition of T cells and/or accessory cells under PLTP-deficient conditions may account for the observed alteration of T-cell polarization. Thus, it has been reported that cholesterol levels can modulate T helper cell orientation both through a direct action on signaling pathways and through a modification of cytokine secretion by dendritic cells.[45–47] As second messengers and activators of protein kinase C, diacylglycerides play an important role at the immunological synapse in the cross talk between T cells and APCs and in T-cell activation.[48] Additionally, the lipid-soluble antioxidant vitamin E, whose distribution is largely dependent on PLTP activity,[7,49–51] is recognized as one of the most effective nutrients that affects immune cell function.[52] With regard to T lymphocytes, studies conducted in old mice and in the elderly suggested that vitamin E can act as an immunostimulating factor through its ability to reduce PGE2 production by accessory cells.[53] Moreover, it has been reported that vitamin E has a direct effect on T-cell functions, by increasing IL-2 production and the proliferation of naive T cells.[54] Unlike cells of the liver and the vascular wall, which contain smaller amounts of vitamin E,[5,8] but similar to circulating erythrocytes, which tend to sequester alpha-tocopherol molecules,[55] spleen mononuclear cells from PLTP-deficient mice have a 2-fold increase in their alpha-tocopherol content compared to WT cells (unpublished data). Whether alpha-tocopherol enrichment of total splenocytes in PLTP-deficient mice contributes to the orientation of $CD4^+$ T cells toward the anti-inflammatory Th2 phenotype deserves further attention.

In conclusion, data from the present work provide the first evidence that PLTP plays a role in modulating the adaptive immune response toward a pro-inflammatory phenotype. This may constitute an additional feature of its pro-atherogenic potency, although it is worthy of note that the immunologic status of the mice was evaluated in the present study under normocholesterolemic conditions (measured plasma cholesterol levels were approximately 100 mg dL^{-1} for WT mice and 75 mg dL^{-1} in $PLTP^{-/-}$ mice), and the relevance of these findings needs to be confirmed in a pro-atherogenic situation, such as in LDL receptor- or apolipoprotein E-deficient conditions.

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COMPETING INTERESTS

The authors declare no financial or commercial conflicts of interest.

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