Lysophosphatidylcholine upregulates CD40 ligand expression in newly activated human CD4+ T cells

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Abstract Lysophosphatidylcholine (lyso-PC) accumulates in tissues undergoing inflammation and atherosclerosis, where an infiltration of T cells is also seen. We found that lyso-PC increased IFN-γ production and CD40L expression in CD4+ T cells stimulated with anti-CD3 Ab and recombinant CD80 molecules, whereas lyso-PC did not affect IL-2 and IL-4 production. These results suggest that lyso-PC, in combination with other stimuli, may regulate CD4+ T cell functions to propagate local inflammatory reactions and also imply a novel role played by a modified lipid in the selection of Th1/Th2 immune response as well as in the T cell mediated pathogenesis in atherosclerosis.

Key words: Lysophosphatidylcholine; CD28-CD80 costimulation; CD4 T cell; CD40 ligand; Atherosclerosis

1. Introduction

Tissue infiltrating T cells are involved in the pathogenesis of such disorders as autoimmunity, inflammation, and infection. Among the infiltrating T cells, CD4+ T cells are important in the sense that they not only recognize class II restricted antigen but also regulate immune response by secreting various lymphokines [1]. In non-classical immune disorders, such as degenerative vascular disorders, CD4+ T cells are also indicated to play indispensable roles [2]. For instance, CD4+ T cell depletion by an anti-CD4 monoclonal antibody reduces post-angioplasty arteriosclerosis in rats by inhibiting the accumulation of mononuclear and smooth muscle cells in the intima of the arterial wall [3], and also reduces the incidence of spontaneous atherosclerosis in hyperlipidemic mice [4]. In the animal model of post-transplantation arteriosclerosis, the blockade of CD28/CD80 mediated T cell stimulation significantly reduced the frequency and severity of arteriosclerosis [5]. Thus, these results indicate that the activated T cells infiltrating the lesion are directly involved in the pathogenesis of these vascular disorders.

The atherosclerotic lesion also sees the accumulation of modified lipids such as ox-LDL [6] and lysophosphatidylcholine (lyso-PC) [7]. However, it is still unknown how the increase of modified lipids in the tissue affects the function of the T cells infiltrating the same tissue. T cells are primarily activated by antigens and costimulation molecules to secrete lymphokines and to express various cell surface molecules. In the milieu, where T cells are activated by antigen presenting cells, the concomitant presence of bioactive lipids may alter the function of those T cells. Considering this, we assessed the effect of lyso-PC, a major phospholipid produced in tissue undergoing infection, inflammation, and atherosclerosis, on the function of the CD4+ T cells which were stimulated through the TCR/CD3 complex and costimulation receptor CD28.

CD40L/CD40 interaction was originally described as regulating B cell growth and differentiation. Stimulation through CD40 augments tumoricidal activity and cytokine production in monocytes and increases antigen presentation by dendritic cells [8]. Recent literature on CD40 includes the control of expression of cell adhesion molecules [9], production of cytokines [10] and matrix degrading enzymes [11] in the EC and SMC in the vascular walls. Here we show the evidence that lyso-PC may enhance the inflammatory response by increasing interferon gamma (IFN-γ) production and CD40 ligand (CD40L) expression in newly activated CD4+ T cells, which could indicate a novel role played by lyso-PC and T cells in the inflammatory aspects of atherogenesis and tissue remodeling in the vascular walls [12].

2. Materials and methods

2.1. LCD32 cells expressing CD80 molecule and cell culture conditions

L cells coexpressing the CD80 and the CD32 molecules were prepared following the method described previously [13]. First, cDNA including the whole coding region of CD80 was obtained by RT-PCR amplification of mRNA extracted from EBV-transformed peripheral B cells using specific primer pairs: sense CCTAAGCATCTGAAGCCATG, and antisense GATGGCAGAATGGAAACATG. Next, the amplified product was cloned into the pCRIII expression vector (Invitrogen) and sequenced. Plasmid DNA from one such clone was transfected with lipofectin (Gibco-BRL, Gaithersbug, MD) into LCD32 cells expressing the FcγII receptor CD32 [14]. The cells were grown in medium containing 400 μg/ml G418 (Life Technology) and HAT (Gibco, Grand Island, NY) to select the stably transfected LCD32 cells. Finally, stable transfectants were screened first for their expression of CD80 using an anti-CD80 Ab (Serotec) and PE-conjugated goat anti-mouse IgG (Caltag), and second for their ability to costimulate T cells pretreated with anti-CD3 Ab using a proliferation assay. The most potent cell line was recloned and designated LCD32/CD80 and used in this study. To control the effect of G418 in the culture, we transfected pCRIII-neo plasmid to LCD32 cells (LCD32/neo). Wild type L cells (Lwt), LCD32/neo and LCD32/CD80 cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FCS (Bio Whittaker), 50 μM 2-mercaptoethanol,

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Abbreviations: CD40L, CD40 ligand; Th1/Th2, T helper type 1/T helper type 2; TCR, T cell receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; LFA-1, lymphocyte function-associated antigen 1; JNK, c-jun N-terminal kinase; PBMC, peripheral blood mononuclear cell

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5% NCTC109 (Gibco, Grand Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin. HAT and G418 were added to the medium for the LCD32/neo and LCD32/CD80 cells.

2.2. CD4+ T cells preparation

T cells were purified from the peripheral blood of healthy donors by E-rosette followed by a Ficoll-Conray method as described elsewhere [15]. CD4+ T were purified by positive selection using a MACS beads cell separation system (Miltenyi Biotec, Sunnyvale, CA). The purity was usually over 90%, which was estimated by flow cytometric analysis using FITC conjugated anti-CD4 (Nu-$T_{H/I}$, Nichirei, Japan) and PE conjugated anti-CD8 (Nu-$T_{S/C}$, Nichirei, Japan).

2.3. Coculture system

For proliferation assays and cytokine analysis, 5×10^4 T cells and 1×10^4 L cells irradiated at 7000 rad were cocultured in 200 μl of RPMI 1640 with 5% FCS. A graded dose of anti-CD3 Ab (ATCC CRL-8001) and lyso-PC (palmitoyl, C16:0, Avanti, Alabaster, AL) were added to the medium. For flow cytometric analysis, 2.5×10^5 T cells and 5×10^4 L cells were cocultured in 1 ml of medium containing 1 μg/ml of anti-CD3 Ab and the indicated amount of lyso-PC. For Northern blot analysis, 5×10^6 T cells and 1×10^6 L cells were cocultured in 10 ml of medium containing 1 μg/ml of anti-CD3 Ab and the indicated amount of lyso-PC.

2.4. Proliferation assay

T cells were cocultured with L cells in flat bottomed 96 well plates (Corning, New York) for 60 h in which the mitogenic effect by an anti-CD3 Ab and a reproducible effect by lyso-PC were best seen. During the last 10 h of culture, 10 μCi [^{3}H]TdR (Amersham, Buckinghamshire, UK) was added to each well; incorporated ^{3}H was measured by a scintillation counter (Top counter, Hewlett-Packard). Cell viability was assessed by trypan blue exclusion at the end of culture. The indicated amount of anti-CD3 Ab and lyso-PC were added to the medium at the start of the coculture.

2.5. Cytokine assay

The supernatants were collected after 36 h of coculture. Interleukin-2 (IL-2) was measured using the CTLL-2 cell line (ATCC, TIB 214) as described elsewhere [16]. IL-4 was measured by proliferation assay using the CT-h4S cell line which expresses the human IL-4 receptor (kindly provided by Dr. William Paul, Laboratory of Immunology, NIAID/NIH) [17]. The detection limit for the IL-4 in this assay was 10 pg/ml. IFN-γ was quantitated by enzyme linked immunosorbent assays (ELISAs) using mouse anti-human IFN-γ (Genzyme) as capture mAb, biotinylated mouse anti-human IFN-γ (Bender MedSystems) and alkaliphosphatase labeled mouse anti-biotin (Zymed) as detection antibodies in 96 well assay plates (Nunc Immuno Plate MaxiSorp). The detection limit for the IFN-γ assay was 1 ng/ml. To control each assay, we used recombinant human IL-2 (Takeda Pharmaceutical Co., Japan), recombinant human IL-4 (Ono Pharmaceutical Co., Japan) and recombinant human IFN-γ (Ootsuka Pharmaceutical Co., Japan).

2.6. Flow cytometric analysis

T cells were stained with optimally diluted PE conjugated mouse anti-human CD2 (Nu-T_{ER}, Nichirei, Japan), biotin conjugated mouse anti-human CD40L (Ancell Corporation), and FITC conjugated anti-CD11a (LFA-1) (Caltag Laboratories). For detection of CD40L, the samples were further incubated with avidin conjugated FITC (Pharmingen). Finally, propidium iodide (PI) was added to each cell suspension so that the gates could be set to exclude dead cells. Surface immunofluorescence was assessed using a flow-cytometer (EPICS XL, Coulter Electronics, Hialeah, FL).

2.7. Northern blot analysis

Northern blot analysis of the CD40L mRNA was performed by a standard method utilizing total RNA isolated from CD4+ T cells cocultured. RNA separated in 0.7% agarose gel were transferred onto a Biodyne nylon membrane and hybridized with random labeled cDNA probes of CD40L (residues 391–831) and β-actin (Clontech, Palo Alto, CA). Jurkat cells [18] and EBV transformed B cell line (EBLB) were used as positive and negative controls, respectively.

2.8. Statistic analysis

Statistic analysis was done using Student's *t*-test.

3. Results

3.1. Coculture with LCD32/CD80 cells augments proliferation of T cells stimulated with anti-CD3

In our initial experiment, we created cell lines to stimulate T cells through CD3 and a costimulation receptor, CD28. The dual expression of CD32 and CD80 molecules in LCD32/CD80 cells was confirmed by flow cytometric analysis (Fig. 1A) and proliferation assay using peripheral T cells (Fig. 1B). As previously reported [14], T cells are activated with a CD3 receptor being cross-linked by an anti-CD3 Ab of which the Fc portion binds to CD32 molecules expressed on LCD32/neo and LCD32/CD80. There was no such mitogenic effect by anti-CD3 in the coculture with Lwt cells which do not express

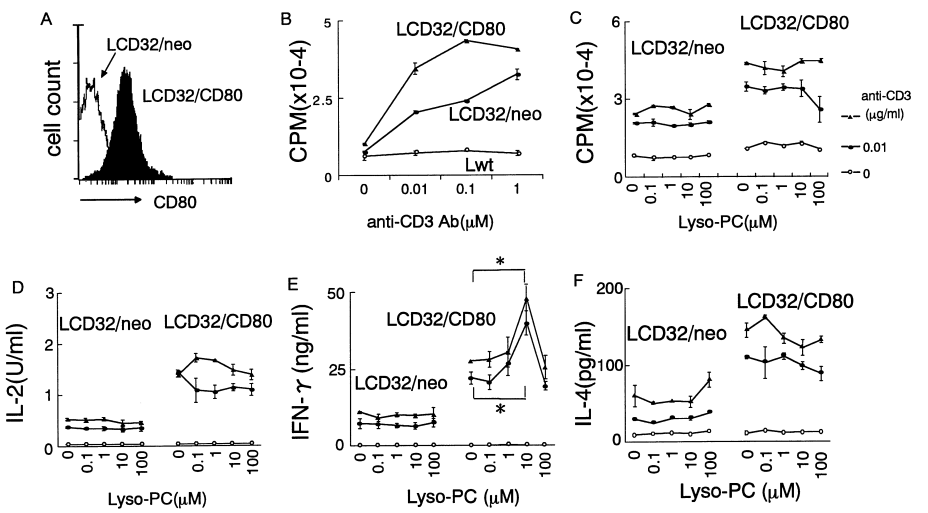

Fig. 1. A: The expression of CD80 in LCD32/neo and LCD32/CD80. B: CD80 molecules expressed on the LCD32/CD80 cells augment anti-CD3 mediated cell proliferation in CD4+ T cells. CD4+ T cells were cocultured with LCD32/neo and LCD32/CD80 in the medium containing anti-CD3 Ab (○, 0 μg/ml; ●, 0.01 μg/ml; ▲, 1 μg/ml) and the indicated amount of lyso-PC. Cell proliferation at 60 h (C), IL-2 (D), IFN-γ (E) and IL-4 (F) secretion at 36 h are shown. Results are expressed as the mean ± S.E.M. (*$P < 0.01$).

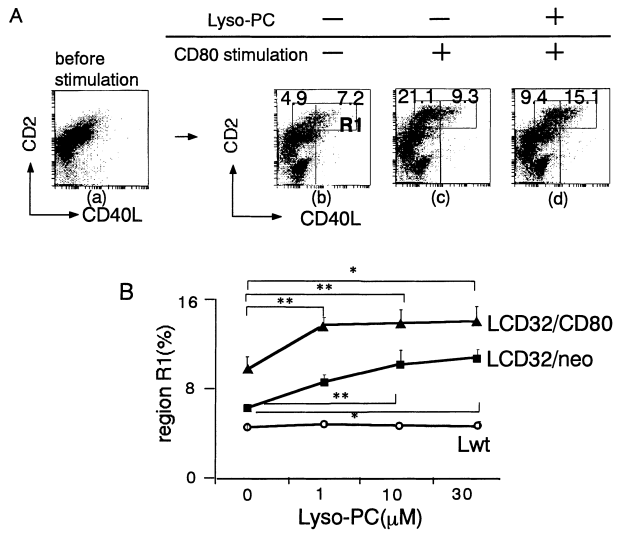

Fig. 2. A: Flow cytometric analysis of CD2 and CD40L expression in CD4+ T cells cocultured with Lwt, LCD32/neo, and LCD32/CD80 for 60 h in the medium containing anti-CD3 (1 μg/ml) and lyso-PC. Purified CD4+ T cells before stimulation (a), cocultured with LCD32/neo (b) and LCD32/CD80 (c). Lyso-PC (30 μM) was added to the medium of coculture with LCD32/CD80 (d). B: The percentage of $CD2^{bright}$ and $CD40L^{high}$ cells (in region R1) in CD4+ T cells cocultured with indicated L cells and anti-CD3 (1 μg/ml) vs. dose of lyso-PC in the medium (*$P < 0.01$, **$P < 0.05$).

CD32 molecules. Coculture of T cells with LCD32/CD80 augmented a mitogenic effect by anti-CD3 Ab (Fig. 1B), which was explained by the greater amount of IL-2 production [19] with LCD32/CD80 than with LCD32/neo (Fig. 1D). Thus, costimulation by CD80 molecules, expressed on the LCD32/CD80 cell, was synergistic to CD3 cross linking, and resulted in an enhanced proliferation of T cells.

3.2. Effect of lyso-PC on the proliferation and lymphokine production by CD4+ T cells

Addition of lyso-PC to the culture medium did not lead to significant change in cell proliferation and IL-2 production in the coculture either with LCD32/neo or with LCD32/CD80 (Fig. 1C,D). Anti-CD3 stimulation alone induced a minimum level of IFN-γ production, however lyso-PC did not affect IFN-γ production in this culture (Fig. 1E). In contrast, coincubation with 10 μM lyso-PC significantly augmented the IFN-γ production of CD4+ T cells cultured with LCD32/CD80 in the presence of anti-CD3 (Fig. 1E) ($P < 0.01$).

Lyso-PC induced dose dependent increases of IFN-γ up to 50 μM (data not shown), however, this effect was not seen at 100 μM of lyso-PC because of the detergent effect of lyso-PC at this concentration. In IL-4 production, costimulation by a CD80 molecule augmented IL-4 production in anti-CD3 stimulated CD4+ T cells (Fig. 1F). The presence of lyso-PC in the medium did not show significant effects on IL-4 production. Thus, lyso-PC per se did not have an induction effect on IFN-γ production, however, lyso-PC did have a synergistic effect on anti-CD3 stimulation in IFN-γ production when costimulation by CD80 was provided.

3.3. Effects of lyso-PC on CD2 and CD40L expression in the activated CD4+ T cells

Having obtained evidence that lyso-PC modifies the effect of anti-CD3 and CD80 stimulation in cytokine production, we next studied the effect of lyso-PC on the expression of a costimulation ligand CD40L in CD4+ T cells. In this study, CD4+ T cells were activated with three different L cells (Lwt, LCD32/neo, LCD32/CD80) in the presence of anti-CD3 (1 μg/ml) and lyso-PC in the medium for 60 h. Anti-CD3 stimulation induced the emergence of $CD2^{bright}$/$CD40L^{high}$ cells (Fig. 2A b) and we could discern three populations in CD2 positive cells: $CD2^{dull}$, $CD2^{medium}$, and $CD2^{bright}$ cells. The addition of CD80 costimulation further increased the population of $CD2^{bright}$/$CD40L^{high}$ cells (Fig. 2A c). In the coculture with LCD32/neo and LCD32/CD80, addition of lyso-PC increased the percentage of $CD40L^{high}$ cells in $CD2^{bright}$ cells (Fig. 2A d, Fig. 2B). In the time-course study, the presence of lyso-PC in the coculture medium facilitated the appearance of $CD2^{bright}$/$CD40L^{high}$ cells at an earlier time point than in the coculture without lyso-PC (data not shown). To confirm upregulation of CD40L molecules by lyso-PC, we did a Northern blot analysis. This clearly indicated that upregulation of CD40L molecules was associated with an increase of their mRNA level. In the coculture of

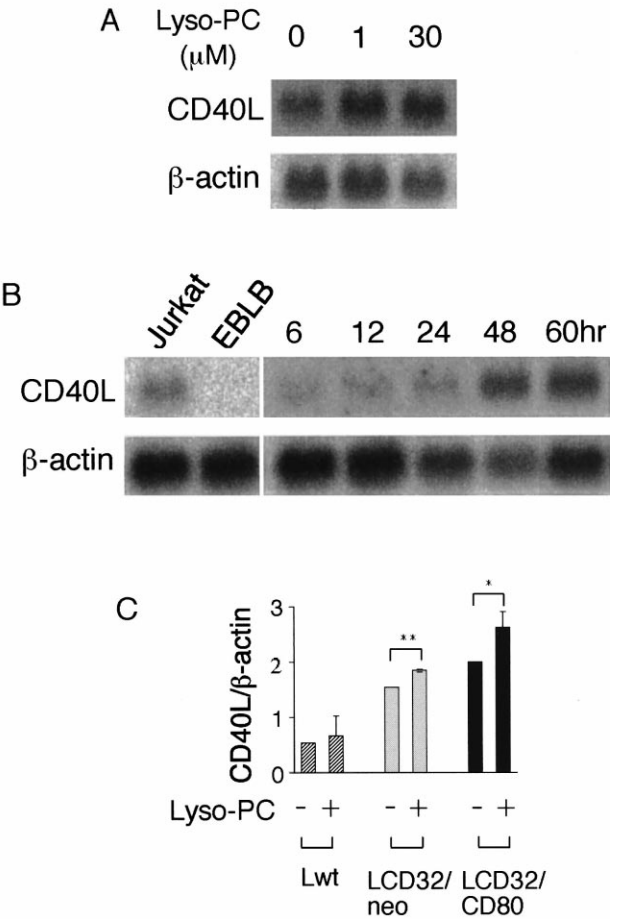

Fig. 3. Northern blot analysis of CD40L expression in CD4+ T cells. A: The dose response of CD40L mRNA synthesis to the concentration of lyso-PC (0, 1, 30 μM) by the CD4+ T cells cocultured with LCD32/CD80 in the presence of anti-CD3 (1 μg/ml). B: The time course of CD40L mRNA synthesis by the CD4+ T cells cocultured with LCD32/CD80 in the presence of anti-CD3 (1 μg/ml) and lyso-PC (30 μM). CD4+ T cells were cocultured with indicated L cells. Stimulation was done using anti-CD3 Ab (1 μg/ml) and lyso-PC (0, 30 μM) for 48 h. C: Density plots of CD40L mRNA signals normalized with β-actin signals (*$P < 0.01$, **$P < 0.05$).

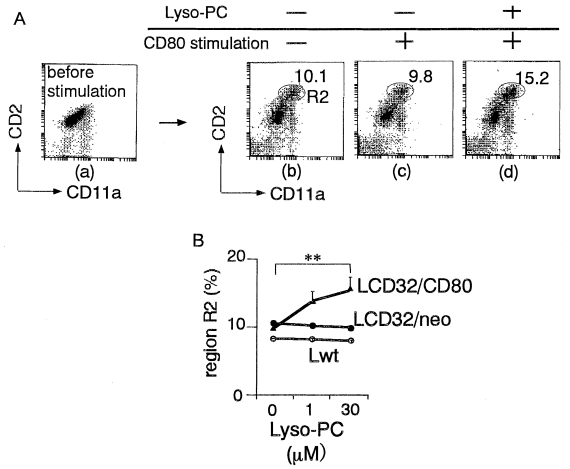

Fig. 4. A: Flow cytometric analysis of CD2 and CD11a (LFA-1) expression in CD4+ T cells cocultured with Lwt, LCD32/neo, and LCD32/CD80 for 90 h. Purified CD4+ T cells before stimulation (a). Cocultured with LCD32/neo (b) and LCD32/CD80 (c) in the medium containing anti-CD3 (1 μg/ml). Lyso-PC (30 μM) was added to the medium of coculture with LCD32/CD80 containing anti-CD3 (1 μg/ml) (d). B: The percentage of $CD2^{bright}/CD11a^{positive}$ cells (in region R2) in CD4+ T cells cocultured with indicated L cells and anti-CD3 (1 μg/ml) vs. dose of lyso-PC in the media ($^{**}P < 0.05$).

CD4+ T cells with LCD32/neo and LCD32/CD80, the presence of 30 μM lyso-PC in the medium augmented the increase of CD40L mRNA level which was induced by anti-CD3 treatment (Fig. 3A,C). This effect by lyso-PC was dose dependent (Fig. 3A), and the maximal effect was seen at 48 h of coincubation (Fig. 3B). Thus lyso-PC stimulation was synergistic to the stimulus provided by anti-CD3 in the upregulation of CD40L at mRNA level.

We next studied the effect of lyso-PC on the expression of CD11a (LFA-1). Lyso-PC did not augment the expression of CD11a in CD4+ T cells stimulated with anti-CD3 alone (Fig. 4B). However, with costimulation by CD80 in addition to anti-CD3 stimulation, we did see an increase of the CD11a expression in the lyso-PC treated CD4+ T cells (Fig. 4A,B).

4. Discussion

In lymphoid organs, T cells are primarily activated by stimuli provided by antigens, cytokines and various cell adhesion molecules. The requirements of costimulation [20] and cytokine stimulation [1] in T cell activation and differentiation are well documented. In non-lymphoid organs, such as vascular walls and intestinal walls, activation and functional differentiation of tissue infiltrating T cells are also affected by a microenvironment containing lipid derived bioactive mediators [21] or bacteria derived lipopolysaccharides [22] which are known to regulate T cell function.

Lyso-PC is catabolized from phosphatidylcholine by the secreted phospholipase A2 (PLA2) [7] or by a free radical reaction [6] in tissue with on-going inflammation [23] and atherosclerotic lesions [24]. In physiological circumstances, T cells are mostly stimulated by the antigen presenting cells which are endowed with various costimulation molecules, most notably CD80/86. Recently the involvement of PLA2 in the CD80/86-CD28 signaling cascade has been demonstrated [25]. These data led us to study the lyso-PC effect on T cells activated by stimuli through a CD28 receptor in conjunction with CD3 receptors.

First, we showed that lyso-PC caused an augmentation of IFN-γ secretion (Fig. 1E) and upregulation of CD40L (Fig. 2) and CD11a expression (Fig. 4) in CD4+ T cells stimulated with anti-CD3 and CD80. Anti-CD3 and anti-CD3/CD80 stimuli were essential in these experiments, since lyso-PC stimulation alone is not potent enough to induce IFN-γ secretion and CD40L/CD11a upregulation.

Our results showed that lyso-PC sufficiently coactivates anti-CD3 stimulated CD4+ T cells in CD40L upregulation (Fig. 2), however, it requires CD80 stimulation for optimal IFN-γ production (Fig. 1) and CD11a upregulation (Fig. 4). This different requirement of CD80 stimulation could be explained in part by the fact that lyso-PC activates JNK [26], which is also activated by the CD28-CD80 costimulatory signal [20,27]. Thus lyso-PC stimulation transduces signals which partly converge to the stimulation conveyed through the CD28 costimulation receptor and provides an additive effect on the costimulation signal necessary for the optimal induction of IFN-γ and CD11a. Although anti-CD3 stimulation is sufficient to upregulate CD40L expression, lyso-PC stimulation could surrogate CD80 stimulation and augment CD40L expression. However, we do not preclude the possibility that lyso-PC transduces signals qualitatively different from those provided by CD80 stimulation, because the presence of lyso-PC stimulation downregulates VLA-4 expression which is upregulated by anti-CD3 and CD80 stimulation (data not shown).

Second, the fact that lyso-PC augmented CD40L expression and IFN-γ production implies two pathophysiological significances. (1) In inflamed tissue, where lyso-PC is synthesized by secretory PLA2 [7] or by active oxygen [6], both CD40L and IFN-γ activate monocytes and macrophages to produce reactive nitrogen intermediates or various monokines [28] which further propagate and sustain the inflammatory reaction. CD40L and IFN-γ also activate antigen presenting cells (dendritic cells) to upregulate class II molecules [29] and costimulation molecules [29], which in turn further activate T cells in an antigen specific manner. In addition, since CD40 is necessary for the generation of Th1-type memory T cells [30,31], this lyso-PC effect in the regulation of CD40L and IFN-γ implies the possible involvement of lyso-PC in the selection of Th1/Th2 response. (2) In atherosclerosis, T cells, migrating to the intima where lyso-PC is synthesized from phosphatidylcholine or ox-LDL, potentially interact with macrophages to enhance its monokine production or interact with CD40 positive SMC to induce chemokine production [10] or alter the proliferation potency of SMC. Alternatively, CD40L positive CD4+ T cells may contribute to the destabilization of plaque and a fibrous cap by inducing matrix degrading enzymes in the SMC through CD40-CD40L interaction [11,12]. Moreover, the possible induction of Th1 cells by lyso-PC may help the production of tissue factor and procoagulant activity by macrophages [32].

Finally, lyso-PC increases the population of T cells expressing CD2 and CD11a (Fig. 4), of which the counter ligand LFA-3 and ICAM-1 are expressed in endothelial cells, suggesting that lyso-PC could regulate the interaction of T cells with endothelial cells which are known to upregulate ICAM-1 by lyso-PC treatment [33]. Taken together, one can envisage that lyso-PC could regulate the T cells' interaction with endo-

thelial cells and thereby possibly regulate the migration of T cells to the intima.

In summary, the present study shows that lyso-PC augments IFN-γ production and upregulates CD40L and CD11a expression in CD4+ T cells. The overall effects of lyso-PC depend on the presence of concomitant activation notably by CD3 and CD28 mediated stimuli. The fact that lyso-PC can induce IFN-γ production and CD40L/CD11a expression in CD4+ T cells explains the novel pathway by which lyso-PC exerts its proinflammatory biological effects, and also suggests other roles played by lyso-PC in Th1/Th2 determination in the immune response and in the pathogenesis of atherosclerosis.

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