

# Low-dose oral cyclophosphamide therapy reduces atherosclerosis progression by decreasing inflammatory cells in a murine model of atherosclerosis

Yayoi Sato-Okabayashi<sup>a</sup>, Kikuo Isoda<sup>a,\*</sup>, Beate Heissig<sup>b</sup>, Tomoyasu Kadoguchi<sup>a</sup>, Koji Akita<sup>a</sup>, Kenichi Kitamura<sup>a</sup>, Kazunori Shimada<sup>a</sup>, Koichi Hattori<sup>c</sup>, Hiroyuki Daida<sup>a</sup>

<sup>a</sup> Department of Cardiovascular Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

<sup>b</sup> Department of Immunological Diagnosis, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

<sup>c</sup> Center for Genomic & Regenerative Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

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## ABSTRACT

**Background:** Atherosclerosis is a chronic inflammatory disease responsible for most cases of heart disease and stroke in Western countries. The cytotoxic drug cyclophosphamide (CPA) can modulate immune functions, and it has therefore been used to treat patients with autoimmune diseases. Extension of survival of patients with severe atherosclerosis has been reported after CPA treatment, but the underlying mechanism is still poorly understood.

**Methods and results:** We have investigated the effects of CPA in a murine model of atherosclerosis. Continuous oral administration of low-dose CPA (20 mg/kg/day) prevented atherosclerosis in apolipoprotein E-deficient (apoE<sup>-/-</sup>) mice fed with a high fat diet. After 12 weeks, CPA treatment delayed progression of atherosclerosis in the mice (9.92% vs 3.32%,  $P < 0.05$ ,  $n = 7$ ) and reduced the macrophage content of plaques (1.228 vs 0.2975 mm<sup>2</sup>,  $P < 0.001$ ). Flow cytometry (FACS) showed that, in peripheral blood and spleen cells, the numbers of B cells and inflammatory T cells (Th1 cells) decreased, and inflammatory monocytes also decreased. However, there were no differences in the bone marrow cells between the two groups. The mRNA levels in the aorta showed significantly decreased inflammatory cytokine (interleukin-6) ( $P < 0.05$ ), and tended to increase anti-inflammatory cytokine (argininase-1), but no significant differences between the two groups. High dose CPA has cardiotoxicity, but the dose used in this study did not show significant cardiotoxicity.

**Conclusions:** The results demonstrate that oral treatment with CPA inhibits initiation and progression of atherosclerosis in the apoE<sup>-/-</sup> mouse model through immunomodulatory effects on lymphoid and inflammatory cells.

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## 1. Introduction

Inflammation participates pivotally in all stages of atherosclerosis, from lesion initiation to progression and destabilization [1,2]. Current treatments for atherosclerosis are mainly based on drugs that lower the plasma cholesterol concentration and blood pressure. In particular, statins have been proven to significantly reduce cardiovascular events, not only as a consequence of their cholesterol-lowering properties but also through their more recently identified anti-inflammatory effects [3,4]. Nevertheless,

atherosclerosis remains the primary cause of heart disease and stroke in Western countries, accounting for up to 50% of deaths. The identification of promising new anti-inflammatory therapies is therefore of great interest.

The development of atherosclerotic plaque continues as T-cells, mast cells, monocytes/macrophages, and other inflammatory cells are recruited to the intima [5]. The most prominent cells that invade into progressing lesions are monocytes/macrophages and T cells. Both types of cells produce many inflammatory cytokines and chemokines, such as interleukin (IL)-6, IL-12, NFκB, and interferon-γ (IFN-γ), and thus create atherosclerosis [5].

T cells play an important role during early atherosclerosis development [6]. It has been shown that cyclophosphamide (CPA) treatment affects the Th1/Th2 balance of activated T cells in patients with autoimmune disease and decreases IFN-γ and

\* Corresponding author at: Department of Cardiovascular Medicine, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.

E-mail address: [kisoda@juntendo.ac.jp](mailto:kisoda@juntendo.ac.jp) (K. Isoda).

IL-12 production by monocytes, whereas it increases secretion of IL-4 and IL-10 by peripheral blood mononuclear cells [7].

CPA, an alkylating cytotoxic drug, has been used extensively as a chemotherapeutic agent against certain solid tumors and lymphomas, and due to its immunomodulatory features, it is in clinical use for the treatment of autoimmune conditions such as multiple sclerosis and rheumatoid arthritis [8,9]. The proposed underlying mechanisms for CPA in autoimmune diseases involve augmentation of immune responses, changing the Th2/Th1 responses due to the “cytokine storm” during the recovery phase, and inhibition of a suppressor cell population [8]. Matar et al. described a Th2/Th1 shift in the cytokine profile of lymphoma-bearing rats occurring with administration of a single low dose of CPA (10 mg/kg, i. p.) [10]. These immunomodulatory properties suggest that CPA might be beneficial in the treatment of atherosclerosis. Indeed, cases of patients with atherosclerosis showing that CPA treatment had extended their survival have been reported [11], but the underlying mechanism is still poorly understood. In this study, CPA treatment was found to delay atherosclerosis progression in apoE<sup>-/-</sup> mice in part due to its immunomodulatory properties.

## 2. Materials and methods

### 2.1. Animals

ApoE<sup>-/-</sup> mice (Jackson Laboratory, ME, USA) were maintained in filtered air Thorensten units. Homozygous apoE<sup>-/-</sup> male mice (8 weeks old; body weight 20–22 g) were fed a high -cholesterol diet (containing 1.25% cholesterol, Clinton-Cybulsky diet, Oriental Yeast, Tokyo, Japan) [12] for 12 weeks. Animal procedures were approved by the Institutional Animal Care and Use Committee of Juntendo University.

### 2.2. Reagents

Cyclophosphamide (Sigma-Aldrich, MO, USA) was dissolved at 0.125 mg/mL in sterilized drinking water and administered orally in the drinking water.

### 2.3. Study design

Mice fed a high fat diet received CPA resuspended in drinking water (20 mg/kg/day p.o.) or water (negative control) for 12 weeks. Retro-orbital blood was collected with a collecting tube (Thermo Fisher Scientific, MA, USA) and white blood cells were counted at specific time points.

### 2.4. Histopathological evaluation of atherosclerotic lesions evaluation

Mice were sacrificed at week 12, and their aorta and heart were perfused with PBS. The aorta was excised and fixed in 4% paraformaldehyde overnight at room temperature and stained with Sudan IV for quantitative analysis of atherosclerotic plaques.

### 2.5. Blood lipid determination

The apoE<sup>-/-</sup> mice were fed a 1.25% cholesterol diet to induce hypercholesterolemia. Blood analysis of plasma lipids (total cholesterol, triglycerides, high-density lipoproteins, and low-density lipoproteins) was performed after overnight fasting at 12 weeks. Plasma samples were stored at -80 °C, and lipids were measured using high-performance liquid chromatography (Sky-light Biotech Inc., Akita, Japan), and other plasma data (lactate dehydrogenase (LDH) and creatine kinase (CK) levels) were also measured (Oriental Yeast).

### 2.6. Histological and immunohistological analyses

Paraffin sections of the aorta to the aortic valve were examined. Paraffin section cut in 3 μm-thick sections and used for hematoxylin and eosin (H&E) staining, Verhoeff's van Gieson (EVG) staining, Masson trichrome (MT) staining, smooth muscle actin (SMA) (Agilent tec (Dako), CA, USA), Mac3 (Becton, Dickinson and Co. (BD), NJ, USA), IL-6 (Abcam, Cambridge, UK), p-NFκB (p65, Bioss, MA, USA) and p-STAT3 (Cell signal, MA, USA) staining. Sections were incubated with a biotylated anti-rabbit or anti-goat secondary antibody (Vector Labs .INC, CA, USA), followed by streptavidin-HRP (Vector labs) and DAB (Vector labs).

### 2.7. Flow cytometry analysis

For analysis of monocytes spleen cells, peripheral blood cells and bone marrow cells were collected. After using Fc blocking material anti-mouse CD16/CD32 solution (BD) cells were stained with anti-CD45-PE-cy7 antibody, anti-Ly6c-PE, anti-CD11b-FITC antibody (Biolegend, CA, USA), anti-CCR2-APC antibody (for spleen cells and peripheral blood cells, R&D systems, MN, USA), anti-CD45R/B220-PE-cy7 antibody (Biolegend) and anti-CD115-APC (for bone marrow cells, Biolegend) and analyzed with FACS Cantoll (BD). Inflammatory (classical) monocytes were identified as CD11b<sup>+</sup> CCR2<sup>+</sup> Ly6C<sup>high</sup> in spleen cells and peripheral blood cells and B220<sup>-</sup>CD11b<sup>+</sup> CD115<sup>+</sup> Ly6C<sup>high</sup> in bone marrow cells [13]. Of the spleen cells used for studying macrophages, inflammatory macrophages (M1 macrophages) were identified as CD45<sup>+</sup>CD11b<sup>high</sup>F4/80<sup>mid</sup>CD206<sup>-</sup>, and anti-inflammatory macrophages (M2 macrophages) were identified as CD45<sup>+</sup>CD11b<sup>mid</sup>F4/80<sup>high</sup>CD206<sup>+</sup> [14]. Total monocyte numbers were calculated by multiplying the total cells by the percent of cells within the monocyte gate. For intracellular studies, spleen cells were isolated, and intracellular cytokines were detected as described previously [15]. Briefly, spleen cells were stimulated for 4 h with 25 ng/mL PMA (Sigma Aldrich) and 1 μg/mL Ionomycin (Sigma Aldrich) and re-suspended to a density of 10<sup>6</sup> cells/mL. Anti-CD4-PE/Cy7 antibody (Biolegend) and anti-CD8a-APC antibody (Biolegend) were used for cell surface staining. Subsequently, the cells were fixed with Cytofix/Cytoperm kit (BD), and intracellular cytokines stained with anti-IFN-γ-FITC antibody (Biolegend) and anti-IL-4-PE antibody (Biolegend) and analyzed with FACS Aria (BD).

### 2.8. Quantitative real-time PCR

Total RNA of whole aorta was isolated with TRIzol reagent (Invitrogen, CA, USA) and PureLink RNA Minikit (Ambion, Thermo Fisher Scientific, MA, USA). Then, 250 ng of RNA were reverse transcribed using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, CA, USA). Quantitative real-time PCR was performed on QuantaStudio3 (Applied Biosystems) using Power SYBR Green PCR master mix (Applied Biosystems). The following oligonucleotide primer pairs were used:

IL-6 (forward): 5'-ACAACCACGGCCTTCCCTACTT-3'  
 IL-6 (reverse): 5'-CACGATTTCCAGAGAACATGTG-3'  
 TNF-α (forward): 5'-GGCAGGTCTACTTTGGAGTCATTGC-3'  
 TNF-α (reverse): 5'-ACATTCGAGGCTCCAGTGAATTCGG-3'  
 IL-10 (forward): 5'-GACCAGCTGGACAACATACTGCTAA-3'  
 IL-10 (reverse): 5'-GATAAGGCTTGGCAACCAAGTAA-3'  
 Arg-1(forward): 5'-CAGAAGAATGGAAGAGTCAG-3'  
 Arg-1(reverse): 5'-CAGATATGCAGGGAGTACC-3'  
 GAPDH (forward): 5'-AAGCCCATCACCATCTTCCA-3'  
 GAPDH (reverse): 5'-GGTCCAGGGTTTCTTACTCC-3'

The housekeeping gene GAPDH was used as an internal control.

## 2.9. Statistical analysis

All data are presented as mean  $\pm$  SEM. Intergroup comparisons were performed by paired Student's *t*-test or analysis of variance (ANOVA). All statistical analyses were performed with GraphPad Prism version 7 (Graph Pad Software). Probability values of  $P < 0.05$  were interpreted to denote statistical significance.

## 3. Results

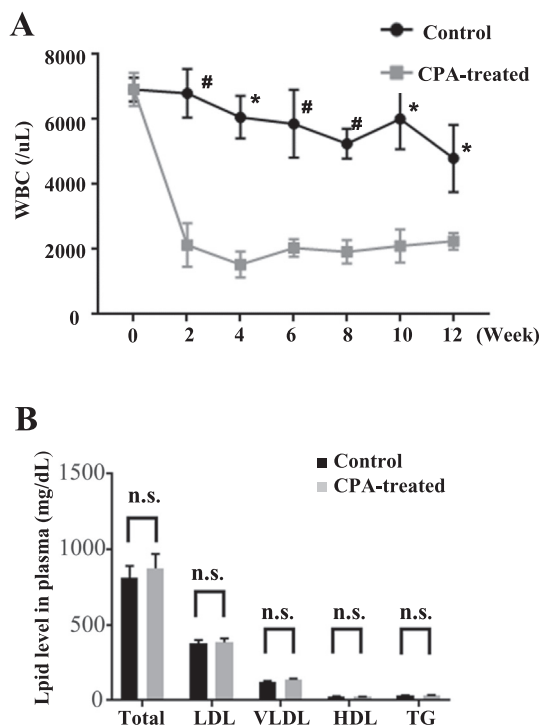
### 3.1. CPA decreased white blood cells in blood with no differences in lipids

After starting CPA, white blood cells (WBCs) started to decrease 2 weeks later, and low WBC levels were maintained for 10 weeks (Fig. 1A, \* $P < 0.005$ , # $P < 0.01$ ,  $n = 6$ ). CPA treatment did not alter blood plasma lipid levels, including total cholesterol, triglyceride and high-density and low-density lipoprotein levels (Fig. 1B, all n.s.,  $n = 6$ ).

### 3.2. Continuous oral administration of CPA prevented atherosclerotic plaque formation in apoE<sup>-/-</sup> mice on a high fat diet

The anti-atherosclerotic potential of CPA was tested in the apoE<sup>-/-</sup> mouse model of atherosclerosis. Preliminary dose-response experiments of CPA on early atherosclerotic lesion development

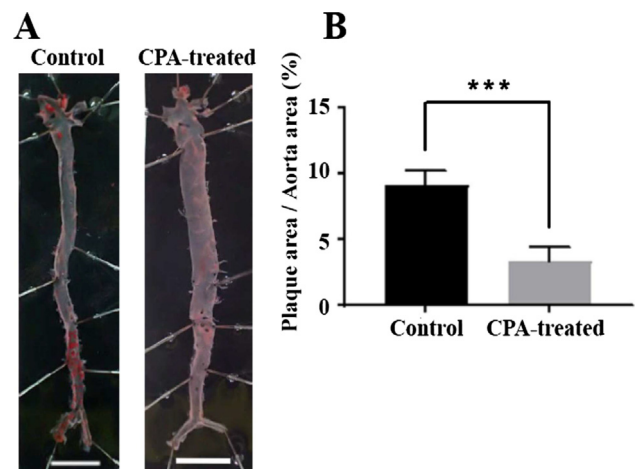
were performed first. The CPA administration dosage was based on studies where even low-dose CPA, with doses ranging from 10 to 40 mg/kg body weight on a daily basis through the drinking water, showed an antitumor effect [12]. The maximal effect of low-dose CPA (from now on referred to as CPA) on reduction of atherosclerotic lesions was observed at a dose of 20 mg/kg per day, with a mild reduction in peripheral leukocyte counts in both apoE<sup>-/-</sup> and apoE<sup>+/+</sup> mice on a high-fat diet (data not shown). The dose of 20 mg/kg CPA was chosen for daily oral administration in order to test its therapeutic efficiency in relation to the development of atherosclerosis (Fig. 2A). In the developed plaques of CPA-treated mice, reduced lipid incorporation was observed (Fig. 2B,  $P < 0.0005$ ,  $n = 7$ ). H&E staining, EVG staining and Masson trichrome staining showed low atherosclerosis areas in the CPA-treated group and fibrotic plaque and non-fibrotic plaque (unstable plaque) in the Control group (Fig. 3A). On EVG staining, plaque area decreased in CPA-treated mice (Fig. 3B,  $P < 0.005$ ,  $n = 6$ ). CPA-treated atherosclerotic lesions were infiltrated by smaller numbers of Mac3-positive macrophages, maintaining SMA construction (Fig. 4A-C, \*\*\*\* $P < 0.0001$ , \* $P < 0.05$ ,  $n = 5-6$ ), suggesting qualitative changes. To show the reduction of IL-6 positive cells in CPA-treated mice, the expressions of p-NF $\kappa$ B and p-STAT3 were checked. Immunological staining with p-NF $\kappa$ B and p-STAT3 showed a decreased positive area of plaque in CPA-treated mice (Fig. 5A-D). The IL-6-positive area (Fig. 5B,  $P < 0.0005$ ,  $n = 8$ ), p-NF $\kappa$ B-positive (Fig. 5C,  $P < 0.001$ ,  $n = 5$ ) and p-STAT3-positive cells (Fig. 5D,  $P < 0.01$ ,  $n = 5$ ) in the plaque area ( were both decreased in CPA-treated mice.



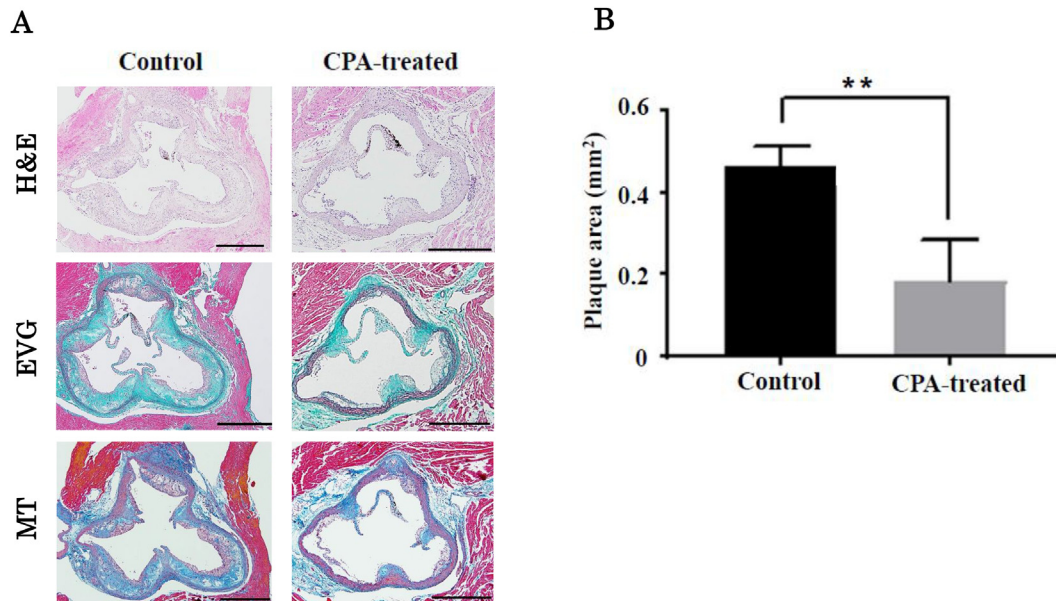
**Fig. 1.** Treatment of low-dose CPA decreased white blood cells, but not change cholesterol profiles in apoE<sup>-/-</sup> mice. (A) Numbers of white blood cells (WBCs) in apoE<sup>-/-</sup> mice treated with high fat diet (HFD) (Control) or HFD with low dose CPA (20 mg/kg/day) (CPA-treated) were checked every 2 weeks. (\* $P < 0.005$ , # $P < 0.01$ ,  $n = 6$  for each group). (B) The levels of total cholesterol (Total), low-density lipoprotein cholesterol (LDL), very low-density lipoprotein cholesterol (VLDL), high-density lipoprotein cholesterol (HDL) and triglyceride (TG) at 12 weeks after treatment of HFD (Control) or HFD with CPA (CPA-treated). (n.s.: not significant,  $n = 6$  per time point).

### 3.3. CPA treatment decreased inflammatory monocytes in peripheral blood cells and spleen cells, but not in bone marrow cells

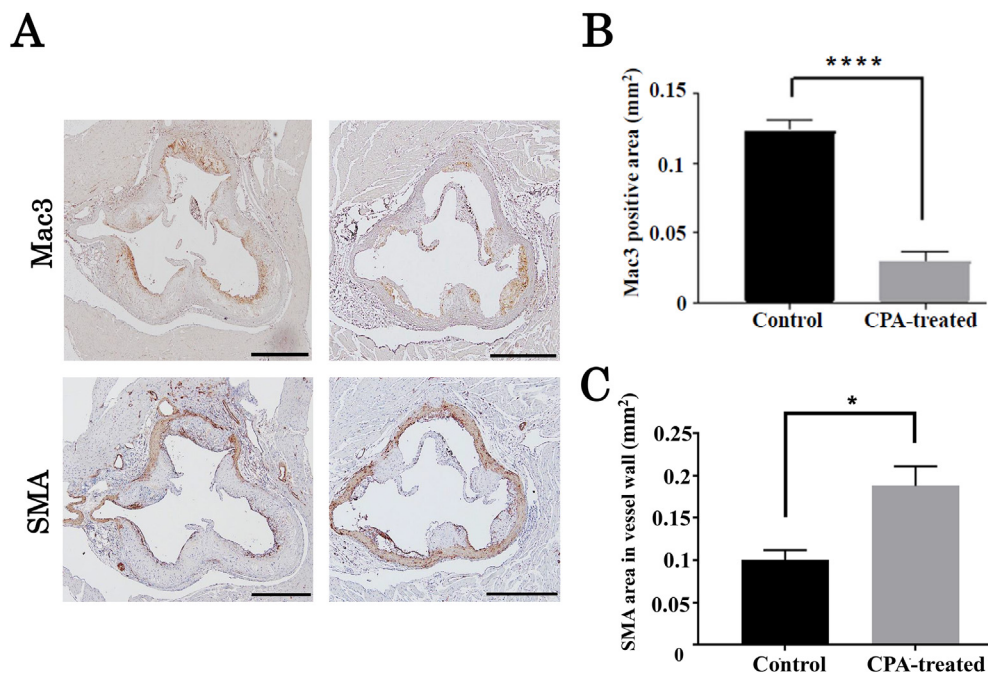
Monocytes in peripheral blood, spleen cells, and bone marrow cells were detected using FACS (Fig. 6A). In peripheral blood, the inflammatory monocytes (CD45<sup>+</sup> Ly6C<sup>high</sup> F4/80<sup>+</sup> CCR2<sup>+</sup>) were fewer in the CPA-treated group than in the control group (Fig. 6B,  $P < 0.001$ ,  $n = 14$ ). Spleen cells showed the same trend as peripheral blood cells (Fig. 6C,  $P < 0.005$ ,  $n = 17$ ). However, bone marrow cells showed no differences between inflammatory mono-



**Fig. 2.** Treatment of low-dose CPA decreased in the extent of atherosclerotic lesions in the aortas (en face) of ApoE<sup>-/-</sup> mice. (A) Sudan IV staining of aortas from two groups at 12 weeks after treatment of HFD (Control) or HFD with CPA (CPA-treated) (bars = 5 mm). (B) Quantitative analysis of the relative surface area of atherosclerotic lesions in aortas. Data are expressed as mean  $\pm$  SEM (\*\*\* $P < 0.0005$ ,  $n = 7$ ).



**Fig. 3.** Treatment with CPA decreased in aortic root atherosclerotic lesions and modulated plaque composition in apoE<sup>-/-</sup> mice. (A) Histological analyses were shown at aortic sinus of apoE<sup>-/-</sup> mice. H&E stains, Elastica van Gieson stains (EVG) and Masson Trichrom stains (MT) were performed. (scale bar = 500  $\mu$ m). (B) Quantitative comparison of the atherosclerotic lesions in the aortic sinus. Data are expressed as mean  $\pm$  SEM (\*\*  $P < 0.005$ ,  $n = 6$ ).



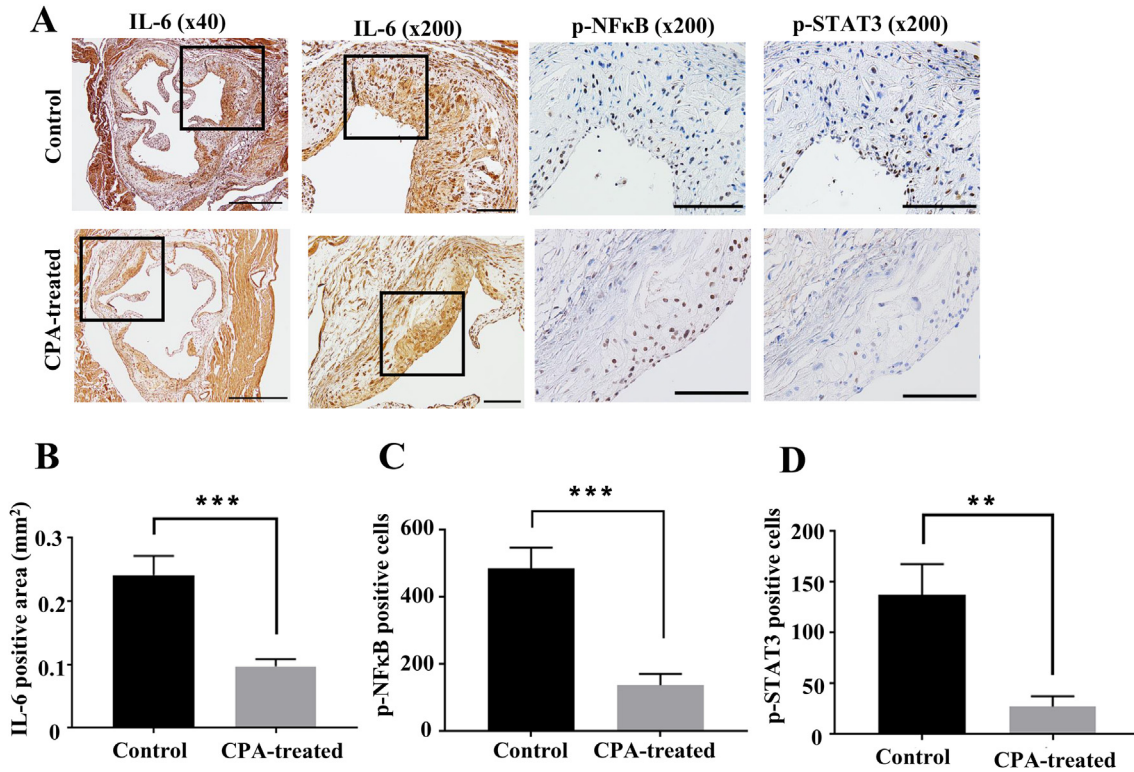
**Fig. 4.** Treatment with CPA decreased accumulation of macrophages and increased vascular smooth muscle cells in the aortic root atherosclerotic lesions of apoE<sup>-/-</sup> mice. (A) Immunohistochemical staining for macrophages (Mac3) and smooth muscle cells (SMA) of aortic root from the two groups at 12 weeks after treatment of HFD (Control) or HFD with CPA (CPA treated). (scale bar = 500  $\mu$ m) Quantitative analysis of Mac3- (B) and SMA-positive (C) area in the aortic root. Data are expressed as mean  $\pm$  SEM (\*\*\*\*  $P < 0.0001$ , \*  $P < 0.05$ ,  $n = 5-6$ ).

cytes (CD45<sup>+</sup> Ly6C<sup>high</sup> F4/80<sup>+</sup> CD115<sup>+</sup>) and anti-inflammatory monocytes (Fig. 6D, n.s.,  $n = 20$ ). Macrophage polarization, inflammatory macrophages (M1; CD45<sup>+</sup> F4/80<sup>+</sup> CD11b<sup>+</sup> CD206<sup>-</sup>) and anti-inflammatory macrophages (M2; CD45<sup>+</sup> F4/80<sup>+</sup> CD11b<sup>+</sup> CD206<sup>+</sup>), was also checked in spleen cells using FACS (Fig. 7A). The data showed that M1 macrophages decreased in the CPA-treated group, but there was no significant difference in M2 macrophages (Fig. 7B, \*\* $P < 0.005$  and n.s.,  $n = 5$ ).

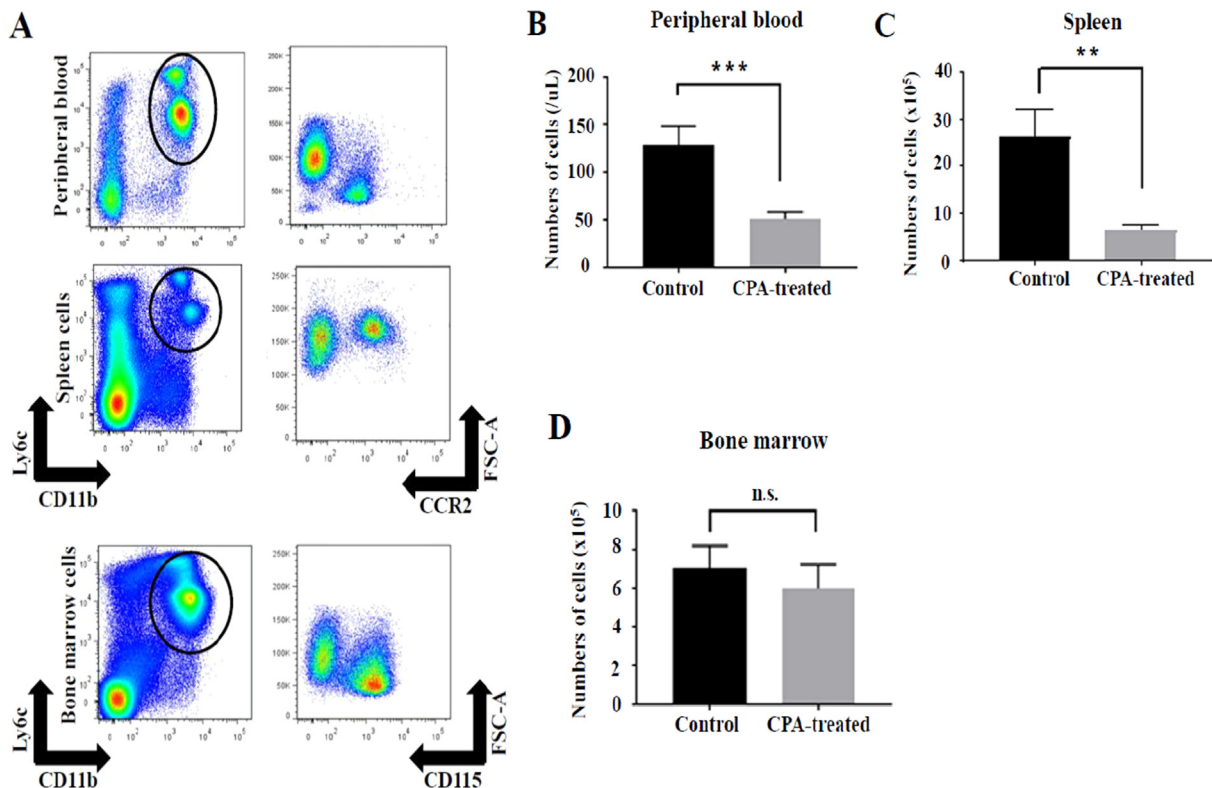
#### 3.4. CPA treatment decreased mRNA of inflammatory cytokines in the aorta

The mRNA levels of the inflammatory and anti-inflammatory cytokines were checked in the aorta at 12 weeks. In the CPA-treated group, the inflammatory cytokines IL-6 and TNF- $\alpha$  tended to decrease (Fig. 8A, \* $P < 0.05$  and n.s.,  $n = 9$  for each), and the anti-inflammatory cytokines IL-10 and Arg-1 tended to increase

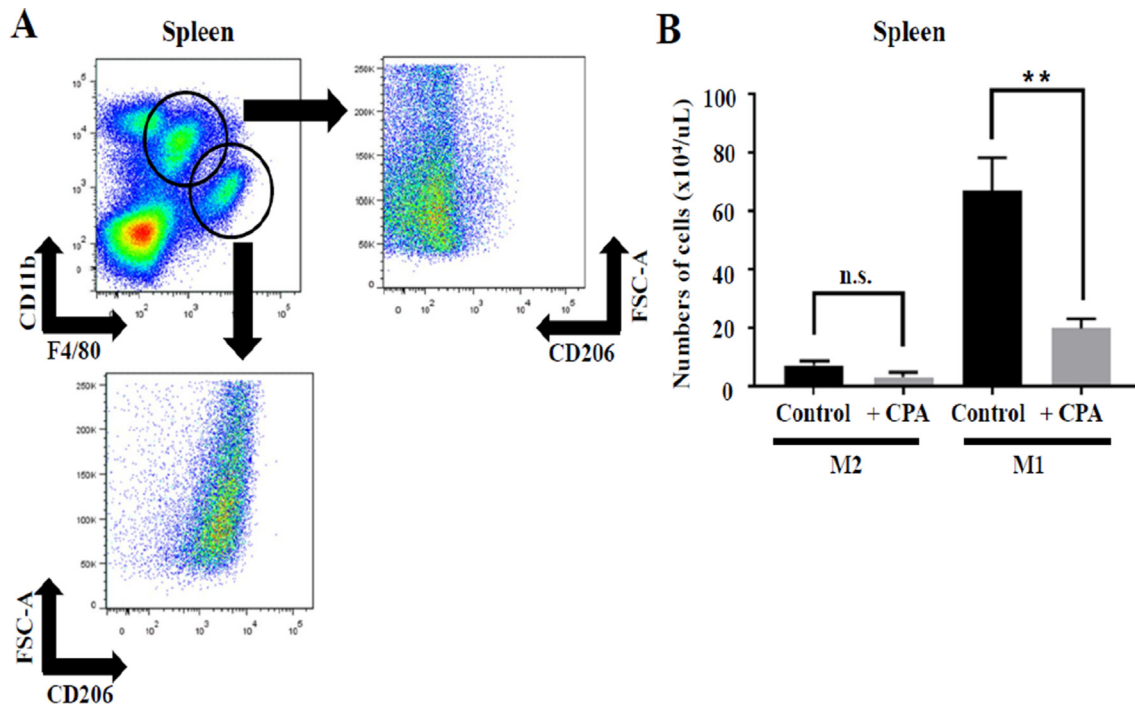




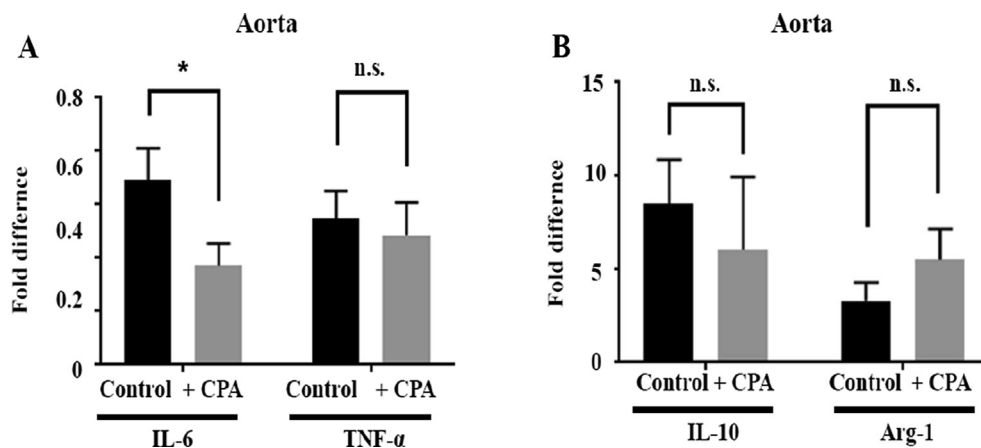
**Fig. 5.** Treatment of CPA significantly inhibited IL-6 expression, NFκB activation, and STAT3 activation in the atherosclerotic lesions of ApoE<sup>-/-</sup> mice. **(A)** Immunohistochemical analyses staining for IL-6, phospho NFκB (**p-NFκB**), and phospho STAT3 (**p-STAT3**) of the aortic root from the two groups at 12 weeks after treatment of HFD (Control) and HFD with CPA (**CPA treated**) (scale bar = 500 μm at x40, =100 μm at x200). Quantitative analyses of IL-6 positive area **(B)**, p-NFκB positive cells **(C)**, and p-STAT3-positive cell **(D)** in vascular wall in section from two groups at 12 weeks after treatment of HFD (**Control**) and HFD with CPA (**CPA treated**). Data are expressed as mean ± SEM (\*\*\*P < 0.001, \*\*P < 0.01, n = 5-8).



**Fig. 6.** Treatment of CPA significantly decreased the numbers of inflammatory monocytes in peripheral, and spleen cells. FACS studies were performed using peripheral cells, spleen cells and bone marrow cells. **(A)** We checked CD11b<sup>+</sup> Ly6c<sup>+</sup> CCR2<sup>+</sup> cells in peripheral cells and spleen cells. In bone marrow cells CD11b<sup>+</sup> Ly6c<sup>+</sup> CD115<sup>+</sup> cells were analyzed. The numbers of inflammatory monocytes in peripheral cells **(B)** (\*\*P < 0.001, n = 14), spleen cells **(C)** (\*\*P < 0.005, n = 17), bone marrow cells **(D)**. Data are expressed as mean ± SEM (n.s.: not significant, n = 20).



**Fig. 7.** Treatment of CPA significantly decreased the numbers of inflammatory macrophages (M1) in spleen. (A) Spleen cells were collected from the apoE<sup>-/-</sup> mice at 12 weeks after treatment of HFD or HFD with CPA. M1 (inflammatory macrophage) and M2 (anti-inflammatory macrophage) were detected by flow cytometry. (B) The numbers of M1 and M2 in spleen cells at 12 weeks after treatment of HFD (Control) or HFD with CPA (+CPA). Data are expressed as mean  $\pm$  SEM (\*\*P < 0.05, n = 5).



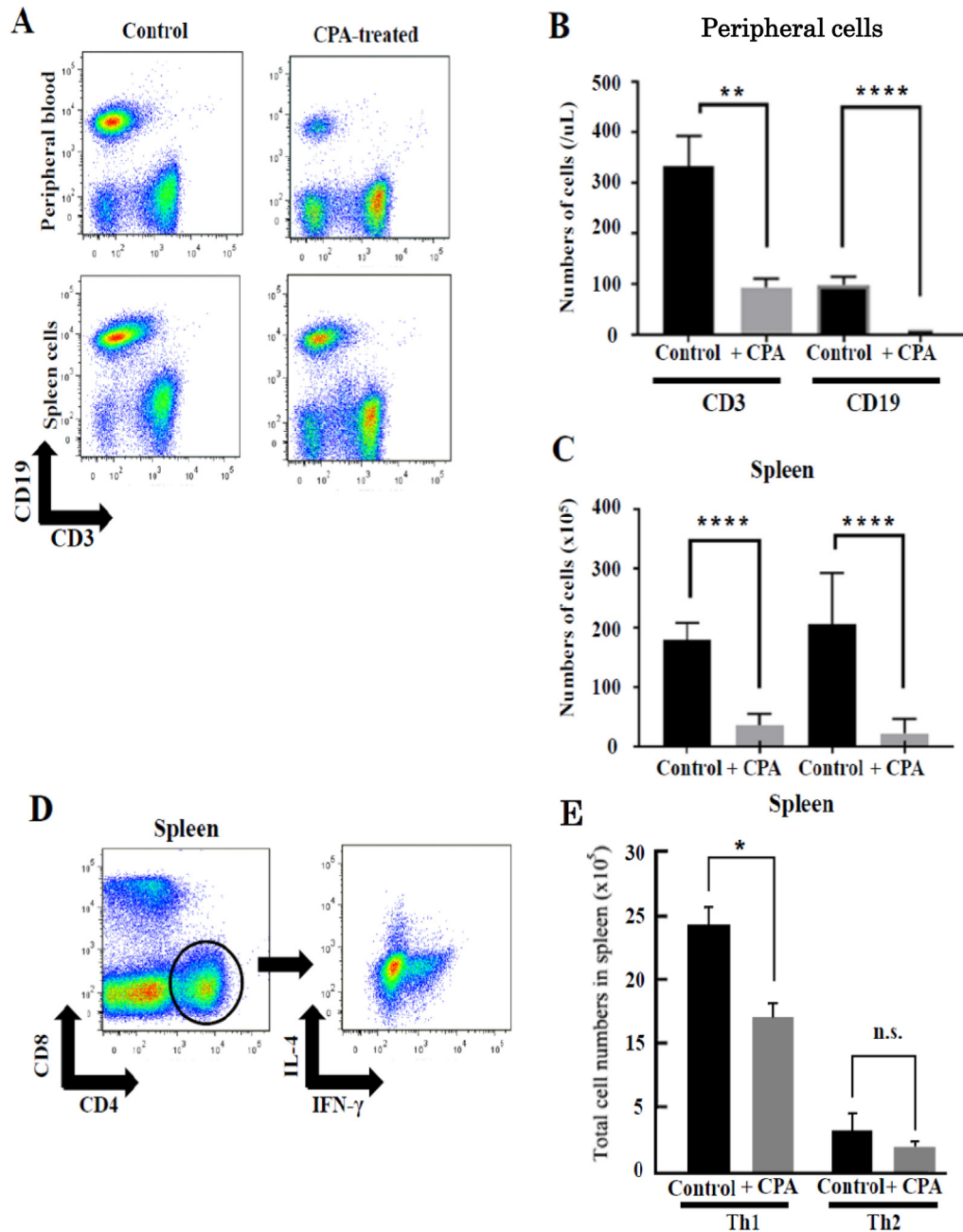
**Fig. 8.** Treatment of CPA significantly decreased IL-6 mRNA expression in the aorta of apoE<sup>-/-</sup> mice. (A) Real-time PCR analysis for mRNA expression levels of IL-6 and TNF- $\alpha$  in the aortas at 12 weeks after treatment of HFD (Control) or HFD with CPA (+CPA). Data are expressed as mean  $\pm$  SEM (\*P < 0.05, n.s.: not significant, n = 9). (B) Real-time PCR analysis for mRNA expression levels of IL-10 and Arg-1 in the aortas at 12 weeks after treatment of HFD (Control) or HFD with CPA (+CPA). Data are expressed as mean  $\pm$  SEM (n.s.: not significant, n = 9).

(Fig. 8B, n.s., n = 9 for each). There was a significant difference in IL-6 between the CPA-treated group and the control group, but other cytokines showed no significant differences.

### 3.5. CPA treatment reduced lymphocytes and inflammatory T cells (Th1)

The FACS study showed decreased CD3-positive T cells and CD19-positive B cells in spleen cells and peripheral blood cells (Fig. 9A-C). CD3-positive T cells and CD19-positive B cells both decreased in CPA-treated mice in the peripheral cells study (Fig. 9B, \*\*P < 0.005, \*\*\*\*P < 0.0001, n = 8-9) and the spleen cells study (Fig. 9C, \*\*\*\*P < 0.0001, n = 12-13). In the peripheral blood study, CD19-positive B cells were more decreased than CD3-

positive T cells. To analyze whether the observed anti-atherosclerotic effects of CPA might in part be mediated by a modified cytokine expression pattern in the CPA-treated mice, the relative ratio of intracellular IFN- $\gamma$  and IL-4 in CD4<sup>+</sup> CD8<sup>-</sup> T-helper cells (Th cells) was assessed by intracellular cytokine staining using FACS (Fig. 9D). There were lower numbers of IFN- $\gamma$ -positive T-helper cells (Th1), whereas the numbers of IL-4-positive T-helper cells (Th2) in the spleen of CPA-treated mice were unchanged, showing that CPA had changed the Th1/Th2 balance towards Th2 in atherosclerosis-prone animals (Fig. 9E, \*P < 0.05, n.s., n = 6). On histological study, CD3-positive T cells were found richly around the aorta and plaque area in the control group, but in the CPA-treated group, CD3-positive cells were few in the plaque area and around the aorta area (Fig. 10A). CD3-positive



**Fig. 9.** Treatment of CPA significantly reduced inflammatory cells and proliferative T cell response and inhibited Th1 polarization. (A) FACS analysis for immune cells was performed using peripheral and spleen cells from apoE<sup>-/-</sup> mice at 12 weeks after treatment of HFD (**Control**) and HFD with CPA (**CPA treated**). We compared with the expression of CD3 and CD19 in the two groups. The numbers of T cells and B cells were analyzed in peripheral cells (**B**) (\*\*P < 0.005 and \*\*\*\*P < 0.0001, n = 8–9) and spleen cells (**C**) (\*\*\*\*P < 0.0001, n = 12–13). Data are expressed as mean ± SEM. (**D**) Th1 and Th2 cells were detected on spleen cells by flow cytometry. Spleen cells were treated for 4 h with PMA and ionomycin, and stained for CD4, CD8, IFN- $\gamma$  and IL-4. (**E**) Relative numbers of IFN- $\gamma$ <sup>+</sup> Th1 cells and IL-4<sup>+</sup> Th2 cells in the pre-gated CD4<sup>+</sup> CD8<sup>-</sup> cell population were determined, and used for calculation of absolute numbers per spleen of mice (\*P < 0.05, n = 3). Data are expressed as mean ± SEM.

cells in the plaque area and the area around the aorta (in the adventitia) were lower in the CPA-treated group than in the control group (Fig. 10B, \*\*\*P < 0.001, \*\*P < 0.05, n = 10).

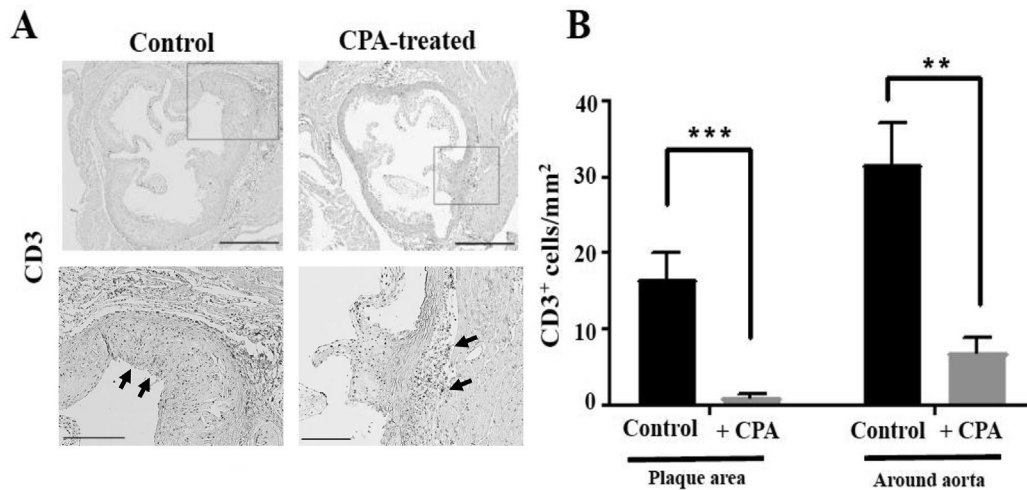
### 3.6. Treatment with low-dose CPA showed no cardiotoxicity in mice

Previous reports showed that CPA was associated with cardiotoxicity and cytotoxicity due to its metabolite acrolein and phosphamide mustard [16,17]. However, other reports demonstrated that low-dose CPA (1–5 mg/kg, p.o.) and its metabolites exert an immunosuppressive effect, whereas a higher dose (120–200 mg/kg, i.v.) exerts a cardiotoxic effect [18–21]. Furthermore,

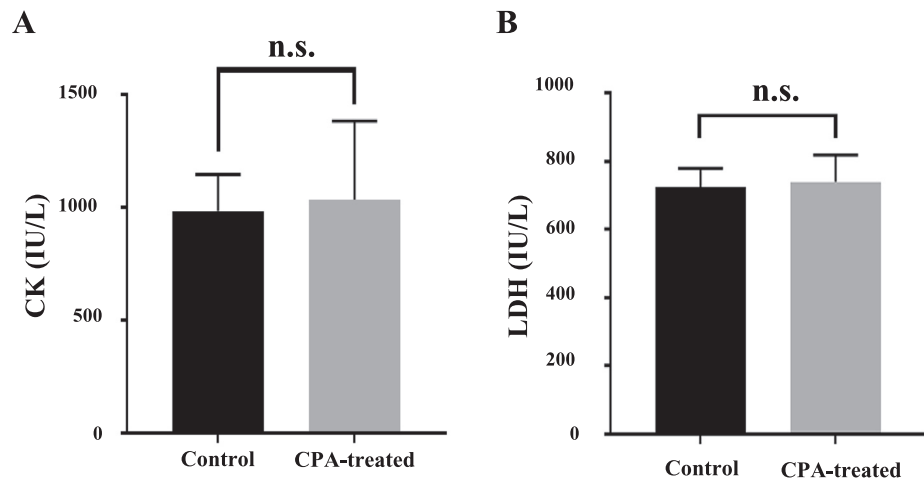
another study showed that CPA administered to rats (30 mg/kg/i. p.) showed CPA-derived acrolein in the aorta [22]. To check for cardiotoxicity, the plasma levels of CK and LDH were checked at 12 weeks in the present study, and there were significant differences in CK and LDH levels between the CPA-treated and control groups (Fig. 11A–B, not significant, n = 9).

## 4. Discussion

The present results showed that administration of low-dose CPA prevented atherosclerosis development in a murine model of



**Fig. 10.** Treatment of CPA significantly decreased in CD3 positive cells recruitment in the plaque and the adventitia. (A) Immunohistochemical staining for CD3 at 12 weeks after treatment of a HFD (Control) and HFD with CPA (CPA treated). Boxed areas are enlarged in the bottom of the panels. Arrows indicate the CD3 positive cells. (scale bar = 500  $\mu$ m at x40 magnification, =200  $\mu$ m at x100 magnification) (B) Results of CD3 positive cell count in the plaque (Plaque area) and the adventitia (Around aorta) at 12 weeks after treatment of a HFD (Control) and HFD with CPA (CPA treated). Data are expressed as mean  $\pm$  SEM (\*\*\*P < 0.001 and \*\*P < 0.005, n = 10 for each).



**Fig. 11.** Treatment of low dose CPA yielded no cardiotoxicity in apoE<sup>-/-</sup> mice. The levels of creatine kinase (CK) (A) and lactate dehydrogenase (LDH) (B) in plasma at 12 weeks after treatment of a HFD (Control) and HFD with CPA (CPA treated). Data are expressed as mean  $\pm$  SEM (n.s.: not significant, n = 9 for each).

atherosclerosis. This anti-atherosclerotic effect was mediated by the immunomodulatory properties of CPA.

CPA or its metabolites damage the bone marrow, thymus, and spleen, leading to decreased B cells and T cells [23,24]. Immune-mediated inflammation is dependent on the counter-regulatory activities of two subpopulations of helper T lymphocytes, Th1 and Th2 cells. The present results provide evidence that the anti-atherosclerotic properties of CPA are associated with a reduction of the Th1 response. IFN- $\gamma$ -producing T helper cells are known to amplify local inflammatory activity by producing pro-inflammatory IFN- $\gamma$ , which contributes to plaque progression [25]. The importance of Th1 cells in the pathogenesis of atherogenesis was demonstrated in studies where administration of recombinant IFN- $\gamma$  or the Th1-cell-inhibiting drug pentoxifylline to hypercholesterolemic mice led to increased and decreased atherosclerosis, respectively [25]. The Th1/Th2 switch has been widely used to ascertain the proatherogenic effect of Th1 and the antiatherogenicity of Th2 [26]. Deficiency of T-bet in Ldlr<sup>-/-</sup> mice, which causes a switch to Th2 and a change in antibody responses, reduced lesion development [27]. In addition, apoE<sup>-/-</sup> mice on a

BALB/c background, which display predominant Th2 responses, showed reduced atherosclerotic lesions at all time points studied [28]. According to these studies, it is said that Th2 has an antiatherogenic effect, and switching Th1 to Th2 causes proatherogenic effects. However, other studies showed that mice deficient in IL-4, which is a signature Th2 cytokine, showed reduced atherosclerosis in a site-specific manner when compared with mice transplanted with IL-4-competent bone marrow [29]. The mice lacking apoE and IL-4 suggest that a proatherogenic role [30].

Previous studies showed that elimination of a large part of the B-cell populations by spleen cells increased plaque formation, whereas adoptive transfer of B cells protected mice from developing disease [31]. Similarly reconstitution of irradiated Ldlr<sup>-/-</sup> mice with B cell-deficient bone marrow has been shown to worsen atherosclerosis [32]. Regulatory B-cells secrete anti-inflammatory cytokines (IL-10 and TGF- $\beta$ ). Strom et al showed that adoptive transfer of a lymph node-derived regulatory B cell-enriched population into apoE<sup>-/-</sup> mice decreased inflammation and atherosclerosis, which depended on IL-10 [33]. However, Sage et al said that B cell-derived IL-10 is not essential to regulate the development of



atherosclerosis [34]. In promoting atherosclerosis, B cells showed ambivalent effects, but most people hypothesized that B cells protect against atherosclerosis. In fact, using anti-CD20 antibody, resulting in a lack of B cell activating factor receptor (BAFFR), protected hypercholesterolemic mice from atherosclerosis [34–36]. Decreasing B2 cells using anti-CD20 antibody further impairs the antibody response to oxidized LDL (ox LDL) and T-cell activation, whereas B1a and plasma cells are not completely depleted by the treatment [37]. In this study, the number of T cells and B cells was reduced, and it is therefore difficult to determine which cells are more effective for anti-atherogenesis.

Monocytes and macrophages play crucial roles in atherosclerosis [38–42]. Atherosclerotic lesions contain a large number of lipid-laden macrophages, known as foam cells, which are derived from circulating monocytes [43]. Mouse monocytes can be divided into at least 2 phenotypically distinct subsets: Ly6C<sup>high</sup>CCR2<sup>+</sup> and Ly6C<sup>low</sup>CCR2<sup>-</sup> [44]. Ly6C<sup>high</sup> monocyte adhered to activated endothelium, infiltrated lesions, and became macrophages in hypercholesterolemic apoE<sup>-/-</sup> mice [45,46]. They also showed that hypercholesterolemia-associated monocytosis developed from increased survival, continued cell proliferation, and impaired Ly6C<sup>high</sup> to Ly6C<sup>low</sup> conversion and subsided with station-induced cholesterol reduction [45]. In the present study, CPA decreased Ly6C<sup>high</sup>CCR2<sup>+</sup> monocytes and prevented the progression of atherosclerosis. A12-week high-fat diet promoted early-phase atherosclerosis, but there were no necrotic areas and fibrotic areas, so that plaque necrosis and regression could not be evaluated.

IL-6 is a key upstream inflammatory cytokine player, propagating the downstream inflammatory response in atherosclerosis [5]. A previous study demonstrated that inflammatory genes, such as IL-6 [47], which is an inducer of STAT3, was highly expressed in atherosclerotic plaques. Moreover, activation of STAT3 has been detected in the plaque [48], and its activation is involved in the progression of atherosclerotic lesions [49]. In the present study, pSTAT3-positive nuclei were analyzed to evaluate the inhibitory effect of CPA in the IL-6 signaling pathway. CPA inhibited STAT3 activation and development of atherosclerotic lesions in apoE<sup>-/-</sup> mice with HFD, suggesting that IL-6 is one of the main contributors to atherogenesis. The present data showed that CPA probably inhibited the p-STAT3-IL-6 pathway through direct or indirect effects.

CPA treatment did not alter lipid levels, and it might be an ideal candidate to combine with drugs known to be gold standard treatments for atherosclerosis, such as statins. The JUPITER study, which was CRP-guided and LDL-guided showed that reduction in cardiovascular events by statin treatment was driven by both LDL lowering and LDL-independent anti-inflammatory effects [50]. Statins have anti-inflammatory, anti-oxidant stress, and lipid reduction effects that are anti-atherogenic [51]. CPA could also induce anti-inflammatory effect, but CPA activates oxidant stress and causes endothelial dysfunction. Thus, the anti-inflammatory effect of CPA appears to be limited, and CPA might be effective for anti-atherogenesis in patients taking statins because of its additional immunosuppressive effect. CPA has been used as a chemotherapeutic agent against certain solid tumors and lymphomas. Furthermore, it is also used for the treatment of autoimmune conditions, such as multiple sclerosis and rheumatoid arthritis (RA), as well as methotrexate. Several anti-inflammatory therapies have been tried for atherosclerotic diseases, but satisfying results have not been obtained [52]. Several reports demonstrated that CPA could improve the vasculitis in patients with autoimmune diseases (i.e. intractable Kawasaki disease and Cherry-Strauss syndrome) [53–55], and improve heart function in SLE patients [56], suggesting that CPA could be useful for the treatment of atherosclerotic diseases in patients.

In the present study, CPA treatment resulted in impaired monocytes/macrophages migration into the plaque. These data are in agreement with the concept that the progression of atherosclerosis results from an imbalance between pro- and anti-inflammatory mediators in response to endothelial injury [47,57].

Major side effects of CPA are pancytopenia, eosinophilia, liver dysfunction, hypogammaglobulinemia, malignant tumor and cardiotoxicity. CPA is one of the nitrogen mustard that is used clinically and metabolized in vivo to phosphoramidate mustard and acrolein (AR). CPA undergoes hepatic metabolism and 4-hydroxy cyclophosphamide (4-HCY) which further metabolizes into aldophosphamide. Aldophosphamide produces phosphoramidate mustard via  $\beta$ -elimination which show anti-cancer activity. Aldophosphamide further metabolizes into non-toxic metabolites like carboxyphosphamide and nitrogen mustard along with toxic compounds AR and AR is mainly responsible for CPA induced cardiotoxicities [16]. CPA and its metabolite exert an immunosuppressive effect at a low dose (1–5 mg/kg, p.o.) whereas higher dose (120–200 mg/kg, i.v.) exert cardiotoxic effect [18–21]. This study, using low dose CPA, showed limited cardiotoxicity, and it suggested benefit using low dose CPA. However, CPA has various side effects, so we should use CPA checking risks and benefits.

The present study also provides the rationale for the positive impact of CPA on atherosclerosis outcomes, since it was shown that CPA, by altering the inflammatory response, changed the composition and generation of atheroma in hypercholesterolemic apoE<sup>-/-</sup> mice without altering hypercholesterolemia. It was also shown that the anti-atherosclerotic effects of CPA were associated with a reduction of the Th1 response and an inhibition of monocyte/macrophage migration to the site of inflammation. Therefore, CPA appears to be a promising novel therapeutic target for pharmacological therapy of early atherosclerotic vascular disease.

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## Declaration of Competing Interest

None declared.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijcha.2020.100529>.

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