CD4$^+$LAP$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ Regulatory T Cells Induced by Nasal Oxidized Low-Density Lipoprotein Suppress Effector T Cells Response and Attenuate Atherosclerosis in ApoE$^{-/-}$ Mice

Yucheng Zhong · Xiang Wang · Qingwei Ji · Xiaobo Mao · Hongxia Tang · Guiwen Yi · Kai Meng · Xiaofang Yang · Qiutang Zeng

Abstract Increasing studies have demonstrated that athero-sclerosis is a chronic immunoinflammatory disease, and that oxidized low-density lipoprotein (oxLDL)-specific T cells contribute to the autoimmune process in atherosclerosis. Oral administration of oxLDL, which was identified as a candidate autoantigen in atherosclerosis, was shown to induce tolerance and suppress atherogenesis. However, the precise mechanisms of mucosal tolerance induction, in particular nasal tolerance, remain unknown. In this study, we explored the effect of nasal oxLDL on atherosclerosis as well as the cellular and molecular mechanisms leading to atheroprotective responses, and then found that nasal oxLDL drastically ameliorate the initiation (47.6 %, $p < 0.001$) and progression (21.1 %, $p = 0.001$) of atherosclerosis. Most importantly, a significant 35.8 % reduction of the progression of atherosclerosis was observed in the enhanced immunization group ($p < 0.001$). These effects were accompanied by a significant increase in CD4$^+$ latency-associated peptide (LAP)$^+$ regulatory T cells (Tregs) and CD4$^+$CD25$^+$Foxp3$^+$ Tregs in spleens and cervical lymph nodes, together with increased transforming growth factor (TGF)-β production and suppressed T-helper cells type 1, 2, and 17 immune responses. Surprisingly, neutralization of TGF-β in vivo partially counteracted the protective effect of nasal oxLDL treatment, indicating that the presence of TGF-β was indispensable to CD4$^+$LAP$^+$ Tregs and CD4$^+$CD25$^+$Foxp3$^+$ Tregs to acquire regulatory properties. Our studies suggest that CD4$^+$LAP$^+$ Tregs and CD4$^+$CD25$^+$Foxp3$^+$ Tregs induced by nasal delivery of oxLDL can inhibit oxLDL-specific T cells response and ameliorate atherosclerosis process.

Keywords Atherosclerosis · immune system · regulatory T cells · tolerance induction · Oxidized LDL

Y. Zhong · X. Wang · X. Mao · G. Yi · K. Meng · X. Yang · Q. Zeng (✉)
Laboratory of Cardiovascular Immunology,
Institute of Cardiology, Union Hospital,
Huazhong University of Science and Technology,
Wuhan 430022, China
e-mail: zengqiutang@gmail.com

Q. Ji
Department of Cardiology, the People's Hospital of Guangxi Zhuang Autonomous Region,
Nanning 530021, China

H. Tang
Department of Infectious and Immunological Diseases, Medical & Health Center for Women and Children, Tongji Medical College, Huazhong University of Science and Technology,
Wuhan 430016, China

Abbreviations

LAP	Latency-associated peptide
ApoE	Apolipoprotein E
oxLDL	Oxidized low-density lipoprotein
Hsp	Heat stock protein
Treg	Regulatory T cell
Th1, 2, 3, and 17	T-helper type 1, 2, 3, and 17
Tr1	Regulatory T-cell type1
Foxp3	Forkhead box P3
CLN	Cervical lymph node
T$_{eff}$	Effector T cell
AChR	Acetylcholine receptor

Introduction

CD4$^+$LAP$^+$ T cell is a unique population of T cells expressing latency-associated peptide (LAP), and having regulatory properties [1, 2]. Recently, human LAP$^+$ Tregs, which are different from classic CD4$^+$CD25$^+$Foxp3$^+$ natural Tregs, have been characterized as a novel population [1]. LAP which is the amino-terminal domain of TGF-β precursor peptide, remains noncovalently associated with TGF-β peptide after cleavage and forms the latent TGF-β complex [2]. Effector T cell response was suppressed by CD4$^+$LAP$^+$ Tregs via a TGF-β-dependent fashion both ex vivo and in vivo [3–6]. Furthermore, these cells have been indicated to inhibit murine autoimmunity in experimental models of autoimmune and inflammatory diseases [4–6] and, importantly, atherosclerotic disease [3].

The concept that atherosclerosis is a vascular wall chronic inflammatory disease mediated by cells of innate and adaptive immunity is no longer controversial [7, 8]. Low density lipoprotein (LDL) retention and oxidation in the subendothelial space promote a chronic inflammatory process with autoimmune responses and subsequently result in atherosclerosis plaques development [9]. Several studies have shown that oxidized LDL (oxLDL)-specific T cells in the vessel wall contribute further to the autoimmune process in atherosclerosis [10], and this autoimmune process may be associated with defects in immunologic tolerance [4]. Thus, strategies to induce immune tolerance are being developed for the treatment of atherosclerosis. One of the principal approaches is mucosal (oral and nasal) administration of autoantigens, which has shown remarkable effects in animal models of atherosclerosis [3, 11–15].

Although oral tolerance induction has been used successfully to protect against a variety of experimental autoimmune diseases, nasal tolerance induction has been identified as more effective approach than the former in inhibiting autoimmune disease because of lacking acid and proteolytic enzymes that are present in the nasorespiratory tract environment [11, 16–20]. Previous studies have demonstrated that CD4$^+$CD25$^+$Foxp3$^+$ Tregs induced by oral oxLDL may be relevant to the suppression of atherosclerosis [13]. In a recent study, Sasaki et al found that oral anti-CD3 antibody treatment inhibits the development of atherosclerosis via a significant increase in CD4$^+$LAP$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ Tregs, which can suppress T-helper cells type 1 and 2 immune responses [3]. Anti-CD3 antibody, however, is not the specific autoantigens in atherosclerosis. Accumulating evidence suggested that different types of antigens could induce a number of different types of Tregs through the nasal and oral routes [3, 13, 15, 19]. Whether nasal administration of autoantigens such as oxLDL could induce CD4$^+$LAP$^+$ Tregs and inhibit atherosclerosis remains uncertain.

In this study, we demonstrate that nasal oxLDL treatment not only effectively inhibits the initiation of atherosclerosis, but also markedly attenuates the progression of atherosclerosis by inducing several types of Treg populations, such as CD4$^+$LAP$^+$ Tregs and CD4$^+$CD25$^+$Foxp3$^+$ Tregs, which suppress effector T cells immune responses in ApoE$^{-/-}$ mice.

Materials and Methods

Mice

Male C57BL/6J ApoE$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred and maintained in the Animal Center of Beijing University. All animal work was approved according to the guidelines of the Care and Use of Laboratory Animals (Science and Technology Department of Hubei Province, China, 2005). Mice were kept under a specific pathogen-free facility and maintained on a 12:12-h light–dark cycle with access to diet and water ad libitum. The mice were fed a normal chow diet during nasal oxLDL treatment. At other times, however, they were given a high-fat diet containing 1.25 % cholesterol and 10 % coconut oil.

Ag and Abs

Human copper-oxLDL was obtained from Yuanyuan Biotechnology of Zhongshan University (Guanzhou, China). In brief, LDL was collected from the plasma of a healthy volunteer [21], and then isolated LDL was oxidized by 10 muM copper sulfate for 24 h at 37°C [22]. Anti-CD3e-APC Ab (clone 145–2 C11), anti-CD4-FITC Ab (clone GK1.5), anti-CD8a-FITC Ab (clone 53–6.7), anti-CD25-PE Ab (clone PC61.5), anti-Foxp3-APC Ab (clone FJK-16 s), anti-IFN-γ-PE Ab (clone XMG1.2), anti-IL-4-PE Ab (clone 11B11), anti-IL-17A-PE Ab (clone eBio17B7), anti-CD3 Ab (clone 17A2), and anti-CD28 Ab (clone 37.51) were obtained from eBioscience (CA, USA). Anti-LAP-APC Ab (FAB2463A) was obtained from R&D Systems (MN, USA). Anti-TGF-β Ab (clone 1D11) and control rat IgG were obtained from ThermoFisher Scientific Inc (Rockford, USA).

Experimental Design

We nasally gave 6-week-old and 16-week-old male mice 15 μL phosphate-buffered saline (PBS, pH=7.4) or 3 μg human copper-oxLDL dissolved in 15 μL PBS by a micropipette daily for 5 consecutive days. Mice were sacrificed by cervical dislocation at 16 and 24 weeks of age, and

atherosclerotic plaques were assessed. In another experiment, 6-week-old mice were nasally administered oxLDL daily for 5 days, and were subsequently injected with 100 μg of neutralizing anti-TGF-β Ab or control rat IgG. Injections were repeated once a week from 7-week-of age to 16-week-of age, and atherosclerotic plaques were assessed.

Measurement of Plasma Total Cholesterol Levels

The plasma was isolated from the blood of each group mice by centrifugation at 1,200× g for 10 min after clotting at room temperature and stored at −60°C. The levels of plasma total cholesterol were determined by autoanalyzer (Hitachi 917).

Tissue Preparation and Atherosclerotic Plaque Assessment

Atherosclerotic plaques were assessed by frozen histological section and en face analysis. The mice were euthanized and perfused with PBS, and then the heart, aorta, and spleen were removed rapidly besides cervical lymph nodes (CLNs), which were regarded as the nose-draining lymph nodes [23]. For aortic root plaque analysis, the heart, including the aortic root, was embedded in OCT compound for cryostat sectioning. 5 consecutive cryosections (10 μm thickness) were prepared from the aortic root containing three aortic valves and stained with oil red O and hematoxylin for lipid visualization. All images were collected and analyzed with the Image-Pro Plus 6.0 software. For en face plaque analysis, the descending aorta was opened longitudinally, and fixed in 4 % paraformaldehyde for 2 h, rinsed and stained with oil red O for 1 h, rinsed again, and paved to a black background surface. Whole vessel images were captured by a digital camera, and analyzed as described previously [24].

Flow Cytometry Analysis of Tregs

Six-week-old mice were nasally administrated oxLDL (3 μg) daily for 5 consecutive days. CLN cells and splenocytes were isolated from untreated mice and oxLDL-treated mice on the 4th and 14th days after the final nasal administration. For the detection of CD4$^+$CD25$^-$LAP$^+$ Tregs, cells were first stained with anti-LAP-APC, followed by anti-CD4-FITC and anti-CD25-PE as described previously [25]. For the detection of CD4$^+$CD25$^+$Foxp3$^+$ Tregs, cells were stained with anti-CD4-FITC and anti-CD25-PE, and then stained with anti-Foxp3-APC after fixation and permeabilization according to the manufacturer's instructions. Isotype controls were conducted to enable correct compensation and

validate antibody specificity. All stained cells were immediately analyzed by FACS Calibur (BD Biosciences).

Flow Cytometry Analysis of Th1, Th2 and Th17 Cells

CLN cells and splenocytes were isolated from the untreated mice and oxLDL-treated mice as described above. For analysis of Th1, Th2 and Th17 cells, cells were suspended at 1.5×10^6 cells/ml in complete RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 % FCS. Cells were stimulated with PMA (20 ng/ml), ionomycin (1 μg/ml) and monensin (2 μmol/l) at 37°C and 5 % CO$_2$ for 4 h. The cells were subsequently harvested and stained with anti-CD3e-APC and anti-CD8a-FITC. After fixation and permeabilization, cells were stained with anti-IFN-γ-PE, anti-IL-4-PE, and anti-IL-17A-PE, respectively. Isotype controls were used to ensure the specificity of the staining. All stained cells were immediately analyzed by FACS Calibur (BD Biosciences).

Cell Purification and Suppression Assays

We purified CD4$^+$ T cells from pooled splenocytes in oxLDL- and PBS-treated mice on the 14th days after the final nasal administration using the CD4$^+$ T cell positive isolation Kit II (Miltenyi Biotec, Gladbach, Germany). Purified CD4$^+$ T cells were stained with anti-LAP-APC Ab followed by anti-CD25-PE Ab, and anti-CD4-FITC Ab. CD4$^+$CD25$^-$LAP$^+$ Tregs and CD4$^+$CD25$^-$LAP$^-$ T cells were isolated by FACS sorting using FACSAria (BD Bioscience) excluding dead and dying T cell subpopulations [22, 26]. CD4$^+$CD25$^+$ Tregs and CD4$^+$CD25$^-$ T cells were isolated from total CD4+ T cells using CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany). The purity of each population was >93 % by FACS analysis. For the detection of suppressive function of CD4$^+$CD25$^-$LAP$^+$ Tregs, purified CD4$^+$CD25$^-$LAP$^+$ Tregs were co-cultured with effector CD4$^+$CD25$^-$LAP$^-$ T cells (5×10^3 cells/well) at different ratios (Tregs/Teff ratios: 1:1, 1:2, 1:4, 1:8) in the presence of soluble anti-CD3 Ab (1 μg/ml) and anti-CD28 Ab (2 μg/ml). For the detection of function of CD4$^+$CD25$^+$ Tregs, purified CD4$^+$CD25$^+$ Tregs were co-cultured with effector CD4$^+$CD25$^-$ T cells (1×10^4 cells/well) at different ratios (Tregs/Teff ratios: 1:1, 1:2, 1:4, 1:8) in the the presence of soluble anti-CD3 Ab (1 μg/ml) and anti-CD28 Ab (1.5 μg/ml). All cells were cultured in a final volume of 150 μl. Cultures were maintained at 37°C with 5 % CO$_2$ for 72 h. The cells were directly pulsed with 0.5 μCi ^{3}H-thymidine for the last 16 h, then the cells were harvested; thymidine incorporation was determined by scintillation counting

(PerkinElmer). Percentage suppression was determined as 1-(cpm incorporated in the co-culture)/ cpm of effector population alone×100 % [26].

Cytokine Assays

Splenocytes were isolated and washed by PBS with 2 % FCS, and then placed in a culture bottle in RPMI 1640 medium for 2 h at 37°C. The non-adhesive cells were collected and cultured at $1×10^6$ cells/well of a 24-wells plate in the presence of 2 μl/mL concanavalin A (ConA). Three days later, the supernatants were collected and quantified analysis by ELISA kits (eBioscience, CA, USA) for TGF-β, IL-10, IFN-γ, IL-4, and IL-17 levels according to the manufacturer's instructions. Additionally, $CD4^+LAP^+$ Tregs were isolated from pooled splenocytes in oxLDL- and PBS-treated mice on the 14th days after the final nasal administration using the same method as described above, and cultured at $1×10^5$ cells/well of a 24-wells plate in the presence of 2 μl/mL ConA. Three days later, the supernatants were collected and measured by ELISA kits (eBioscience, CA, USA) for TGF-β levels according to the manufacturer's instructions. The sensitivity of ELISA kits for TGF-β, IL-10, IFN-γ, IL-4, and IL-17 was 9 pg/ml, 5 pg/ml, 5.3 pg/ml, 2 pg/ml, and 1.6 pg/ml, respectively; and no cross-reactivity was found in detection. All samples were measured in triplicate.

Real-Time RT-PCR Analysis

Total RNA was extracted with RNAiso Plus (Takara Biotechnology, Japan) from Splenocytes and the descending aortas. cDNA was transcribed from purified RNA using RNA PCR Kit (Takara). Real-time PCR was conducted using One Step SYBR Green Mix (Takara) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. Melting curves established the purity of the amplified band after 40 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C. Primer pairs are shown in Table 1. Data are presented and normalized to GAPDH. Amplification reactions were performed in duplicate and all mRNA expression levels were calculated using the comparative CT method formula $2^{-\Delta\Delta ct}$.

Statistical Analysis

All statistical analyses were performed with SPSS 13.0. Data are expressed as means ± SD. ANOVA with Newman-Keuls test was used to compare the data among groups. P-value of <0.05 was considered to indicate significance.

Table I Sequences of primers for real-time RT-PCR

| Molecule	Sequence (5′-3′)
VCAM-1 sense	CCTCACTTGCAGCACTACGGGCT
VCAM-1 anti-sense	TTTTCCAATATCCTCAATGACGGG
MCP-1 sense	TTCCTCCACCACCATGCAG
MCP-1 anti-sense	CCAGCCGGCAACTGTGA
IFN-γ sense	CGGCACAGTCATTGAAAGCCTA
IFN-γ anti-sense	GTTGCTGATGGCCTGATTGTC
IL-4 sense	TCTCGAATGTACCAGGAGCCATATC
IL-4 anti-sense	AGCACCTTGGAAGCCCTACAGA
IL-10 sense	GACCAGCTGGACAACATACTGCTAA
IL-10 anti-sense	GATAAGGCTTGGCAACCCAAGTAA
IL-17 sense	TCTCTGATGCTGTTGCTGCT
IL-17 anti-sense	CGTGGAACGGTTGAGGTAGT
TGF-β sense	GTGTGGAGCAACATGTGGAACTCTA
TGF-β anti-sense	TTGGTTCAGCCACTGCCGTA
CD25 sense	CTGATCCCATGTGCCAGGAA
CD25 anti-sense	AGGGCTTTGAATGTGGCATTG
Foxp3 sense	CTCATGATAGTGCCTGTGTCCTCAA
Foxp3 anti-sense	AGGGCCAGCATAGGTGCAAG
GAPDH sense	ACCACAGTCCATGCCATCAC
GAPDH anti-sense	TCCACCACCCTGTTGCTGTA

Results

Nasal Administration of oxLDL Inhibits the Initiation of Atherosclerosis

To evaluate the effect of nasal oxLDL on the initiation of atherosclerosis, we nasally treated 6-week-old male $ApoE^{-/-}$ mice with PBS (group I) or oxLDL (group II) daily for 5 consecutive days, and then a high-fat diet was given. At 16 weeks of age, the mice were euthanized. Cryosections of the aortic root were stained with Oil Red O and Hematoxylin, and then analyzed quantitatively. Surprisingly, a significant 47.6 % reduction in atherosclerotic lesion size in the aortic root was found in group II compared to group I (Fig. 1A, B, E; 419,459.8±52,532.2 μm^2 vs 219,777.6±14,226.4 μm^2; $p<0.001$). Simultaneously, en face analysis of the descending aortas was performed. A significant 34.1 % reduction in aortic plaque burden was observed in group II compared to group I (Fig. 1C, D, F; 26.7±2.7 % vs 17.6±2.3 %; $p<0.001$).

Nasal Administration of oxLDL Ameliorates the Progression of Atherosclerosis

Next, to evaluate the effect of nasal oxLDL on the progression of atherosclerosis, the initial formation of plaques in the aortic root of 6-week-old mice was induced by feeding a high-fat diet for 10 weeks, and then these mice were nasally

Fig. 1 Nasal oxLDL inhibits the initiation of atherosclerosis. A and B, Representative photomicrographs show Oil Red O and hematoxylin stained aortic root sections from group I (n=8), group II (n=8), respectively. E, Data from group I and II are shown: black circles represent animals from group I, white circles represent animals from group II. C and D, Representative photomicrographs show Oil Red O stained the descending aortas from group I and II. F, Data from group I and II are shown: black and white circles represent animals from group I and II, respectively. A black bar represents 200 μm. Horizontal bars represent means
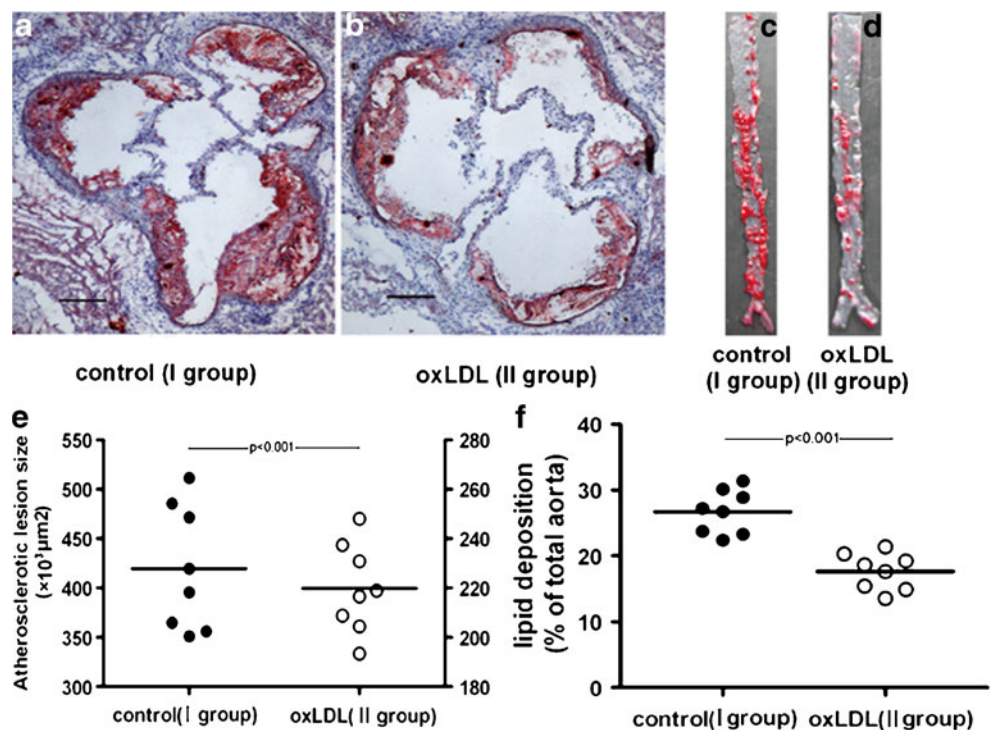
treated with PBS (group III) or oxLDL (group IV) daily for 5 consecutive days, and a high-fat diet was subsequently continued for another 7 weeks. At 24 weeks of age, the mice were euthanized. Atherosclerotic plaques were assessed as described above. OxLDL-treated mice resulted in a moderate but significant 21.1 % reduction in atherosclerotic lesion Crown Prosecution Service, websitesize compared with PBS-treated mice (Fig. 2A, B, G; 698,554.7±56,873.1 μm² vs 545,822.7± 42,467.2 μm²; p=0.001). Furthermore, we found a statistical 15.8 % reduction in aortic plaque burden in oxLDL-treated

mice than in PBS-treated mice (Fig. 2D, E, H; 44.3±4.7 % versus 37.3±3.8 %; p=0.02). In addition, to explore a better method of inhibiting the progression of atherosclerosis, we conducted another experiment. Based on group IV, mice were nasally given 3 μg/dose oxLDL (group V, called the enhanced group) once a week from 18-week-old to 24-week-old, and then sacrificed. Interestingly, we found a significant 35.8 % reduction in atherosclerotic lesion size in group V compared with group III (Fig. 2C, G; 448,749.9±46,296.8 μm²; p< 0.001), and a 17.8 % reduction in group V compared with

Fig. 2 Nasal oxLDL inhibits the progression of atherosclerosis. A through C, Representative photomicrographs show Oil Red O and hematoxylin stained aortic root sections from group III (n=8), group IV (n=8), and group V (n=8). G, Data from three groups are shown: black, half black, and white circles represent animals from group III, IV, and V, respectively. A black bar represents 200 μm. D through F, Representative photomicrographs show Oil Red O stained the descending aortas from group III, IV, and V. H, Data from three groups are shown: black, half black and white circles represent animals from group III, IV, and V, respectively. Horizontal bars represent means
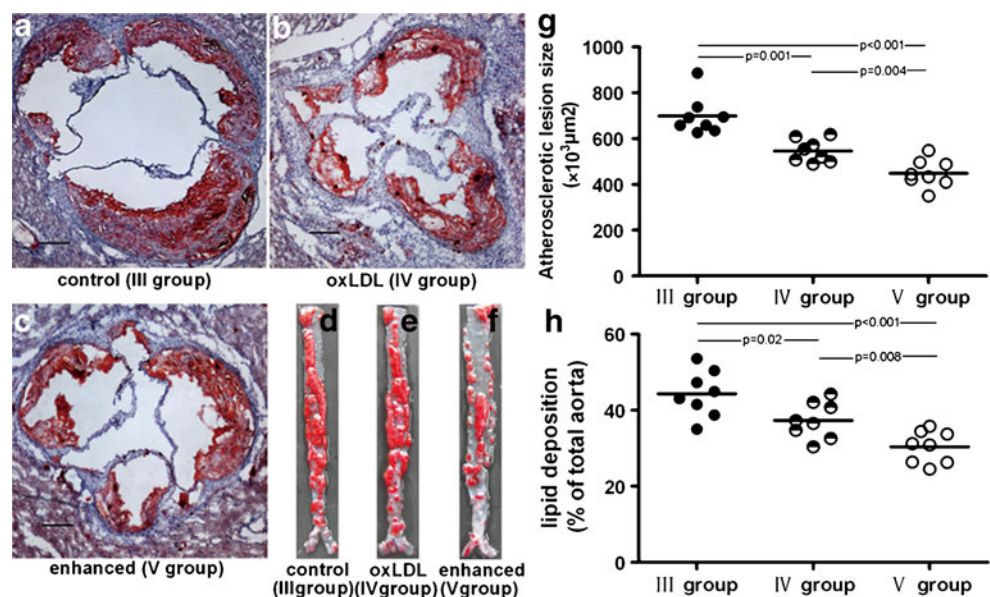
group IV (Fig. 2G, $p=0.004$). Simultaneously, we observed a significant 31.4 % reduction in aortic plaque burden in group V than in group III (Fig. 2G, H; 30.4 ± 3.5 %; $p<0.001$), and an 18.5 % reduction in group V than in group IV (Fig. 2H, $p=0.008$).

### Effect of Nasal Administration of oxLDL on Body Weight and Plasma Lipid

Body weight and plasma total cholesterol levels were similar in group I and II, or in group III, IV, and V, respectively (Table S1). These findings indicated that nasal administration of oxLDL does not markedly affect body weight and plasma total cholesterol levels.

Nasal oxLDL Induces CD4$^+$LAP$^+$ Tregs and CD4$^+$CD25$^+$Foxp3$^+$ Tregs in Spleens and CLNs

To investigate whether nasal oxLDL results in a change in various types of Tregs levels, oxLDL-treated mice were euthanized on the 4th and 14th days after the last nasal treatment, consistent with previous reports [13, 14]. Untreated mice were considered as a control. Using FACS analysis, our data showed that in untreated mice, the number of CD4$^+$LAP$^+$ Tregs and CD4$^+$CD25$^+$Foxp3$^+$ Tregs present in spleens (2.16 ± 0.10 %, 0.94 ± 0.13 %; respectively) and CLNs (4.2 ± 0.33 %, 3.03 ± 0.21 %; respectively) are normal and low. Compared with untreated mice, the number of CD4$^+$LAP$^+$ Tregs and CD4$^+$CD25$^+$Foxp3$^+$ Tregs were increased dramatically to 3.66 ± 0.11 % and 1.56 ± 0.12 % in spleens (Figs. 3A and 4A; all $p<0.01$) and to 9.04 ± 0.40 % and 7.09 ± 0.35 % in CLNs (Figs. 3B and 4B; all $p<0.01$) on day 4, and to 5.21 ± 0.18 % and 2.02 ± 0.21 % in spleens (Figs. 3A and 4A; all $p<0.01$) and to 6.74 ± 0.24 % and 4.71 ± 0.27 % in CLNs (Figs. 3B and 4B; all $p<0.01$) on day 14, respectively. Particularly, we observed a significant increase in the percentage of CD4$^+$CD25$^-$LAP$^+$ Tregs in the CD4$^+$ T cells in spleens and CLNs (Fig. 3D, all $p<0.01$) while no effect was observed in CD4$^+$CD25$^+$LAP$^+$ T cells (data not shown).

In addition, to determine whether nasal oxLDL affects the suppressive function of Tregs, we conducted in vitro suppression assays. Our experiment indicated that the suppressive function of CD4$^+$CD25$^-$LAP$^+$ Tregs and CD4$^+$CD25$^+$ Tregs were not markedly changed by nasal oxLDL (Figure S1A, B; all $p>0.05$). Of note, nasal oxLDL treatment led to a
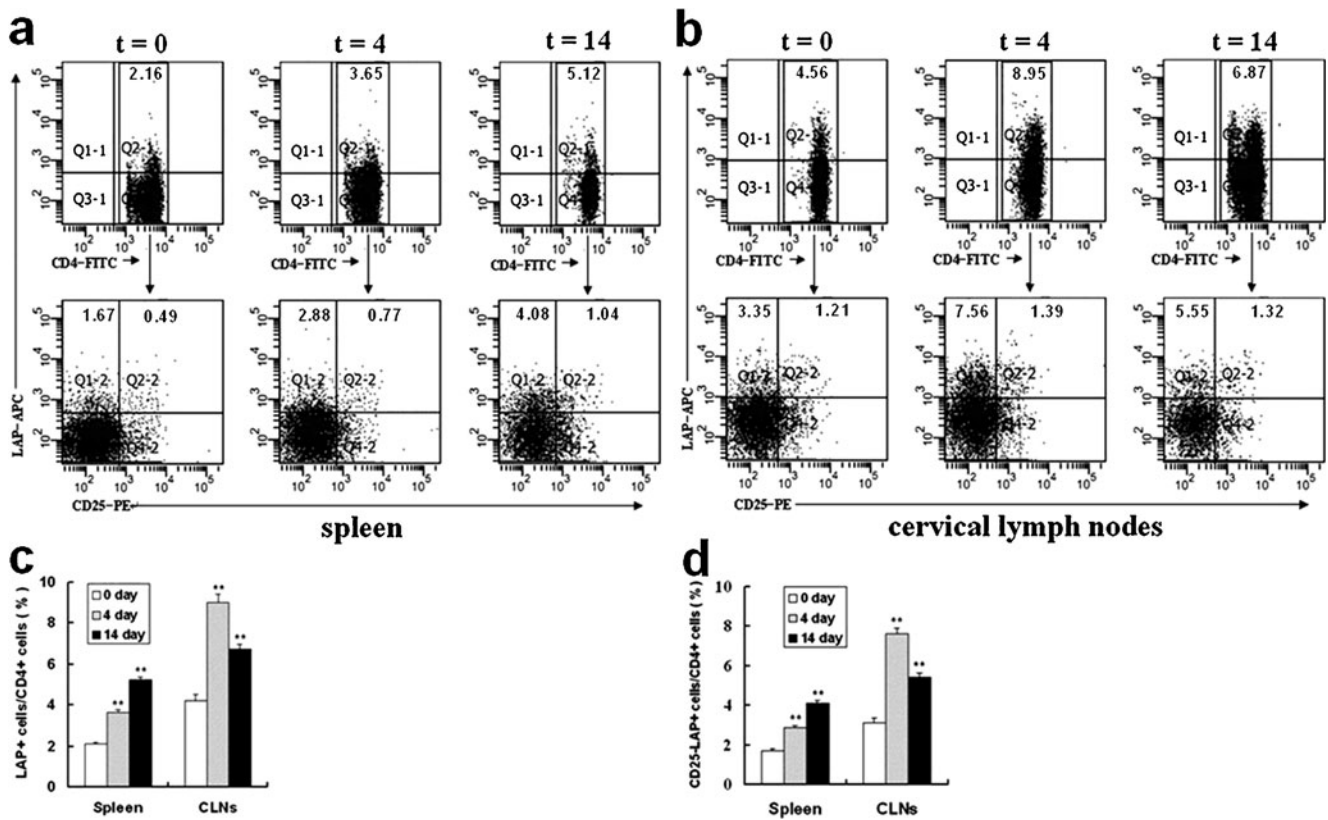
Fig. 3 Nasal oxLDL induced CD4$^+$LAP$^+$Tregs in spleens and CLNs. ApoE$^{-/-}$ mice were nasally given with oxLDL daily for 5 days and were killed at days 4 and 14. Untreated mice were considered as a control. A and B, Representative results of CD4, CD25, and LAP expression in spleens and CLNs estimated via FACS analysis, respectively. C and D, The graphs represent the percentage of CD4$^+$LAP$^+$ Tregs and CD4$^+$CD25$^-$LAP$^+$ Tregs in spleens and CLNs, respectively. $^{**}P<0.01$. Data are mean ± SEM and are representative of at least three independent experiments
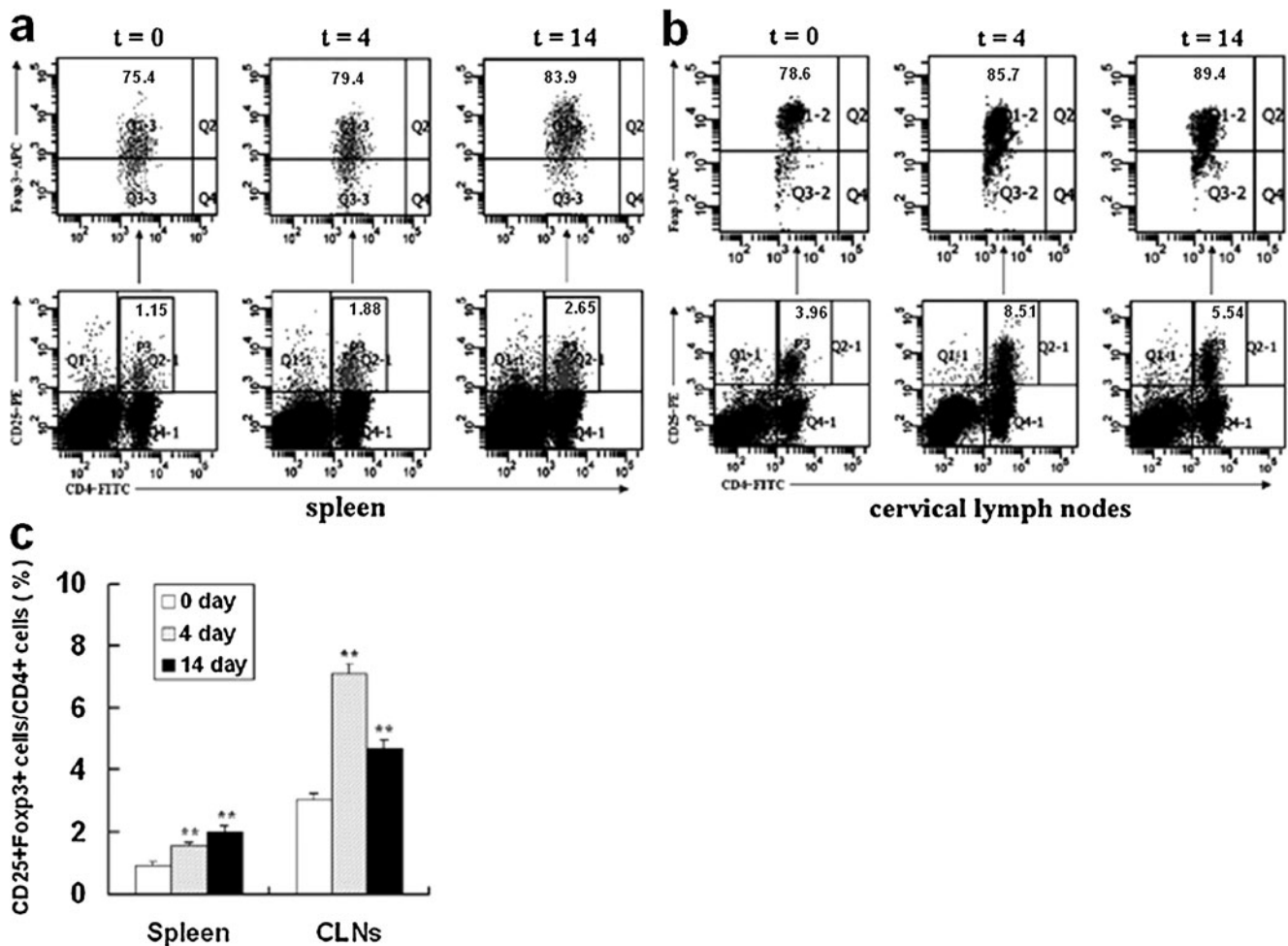
Fig. 4 Nasal oxLDL induced CD4$^+$CD25$^+$Foxp3$^+$Tregs in spleens and CLNs. ApoE$^{-/-}$ mice were nasally given with oxLDL daily for 5 days and were killed at days 4 and 14. Untreated mice were considered as a control. A and B, Representative results of CD4, CD25, and Foxp3 expression in spleens and CLNs estimated by FACS analysis, respectively. C, The graphs represent the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in spleens and CLNs, respectively. **$P<0.01$. Data are mean ± SEM and are representative of at least three independent experiments

significant up-regulation of TGF-β which secreted by CD4$^+$LAP$^+$ Tregs compared with nasal PBS treatment (Fig. 5, $p<0.01$).

Nasal oxLDL Suppresses Th1, Th2, and Th17 Cells in Spleens and CLNs

Meanwhile, we evaluated the effect of nasal oxLDL on Th1, Th2, and Th17 cells in spleens and CLNs. As shown in Fig. 6, there was a significant decrease in the number of Th1, Th2, and Th17 cells in spleens and CLNs of oxLDL-treated mice on day 4 (untreated group vs oxLDL-treated group; 15.3± 2.30 % vs 11.9±0.93 %, 1.27±0.20 % vs 0.93±0.17 %, 1.45± 0.25 % vs 0.83±0.20 % in spleens, all $p<0.05$; 12.5±1.52 % vs 6.38±1.25 %, 0.78±0.12 % vs 0.28±0.12 %, 0.98±0.28 % vs 0.25±0.18 % in CLNs, all $p<0.01$). Similarly, the number of Th1, Th2, and Th17 cells in spleens and CLNs of oxLDL-treated mice were markedly diminished on day 14 (untreated
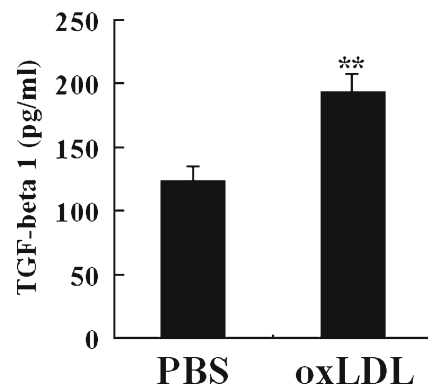
Fig. 5 Effects of nasal oxLDL on TGF-β production in CD4$^+$LAP$^+$ Tregs. CD4$^+$LAP$^+$ Tregs isolated from spleens on 14th day after the last nasal oxLDL and PBS treatment were stimulated with ConA in vitro for 72 h. TGF-β production in supernatants was determined. **$P<0.01$. Data are mean ± SEM and are representative of at least three independent experiments
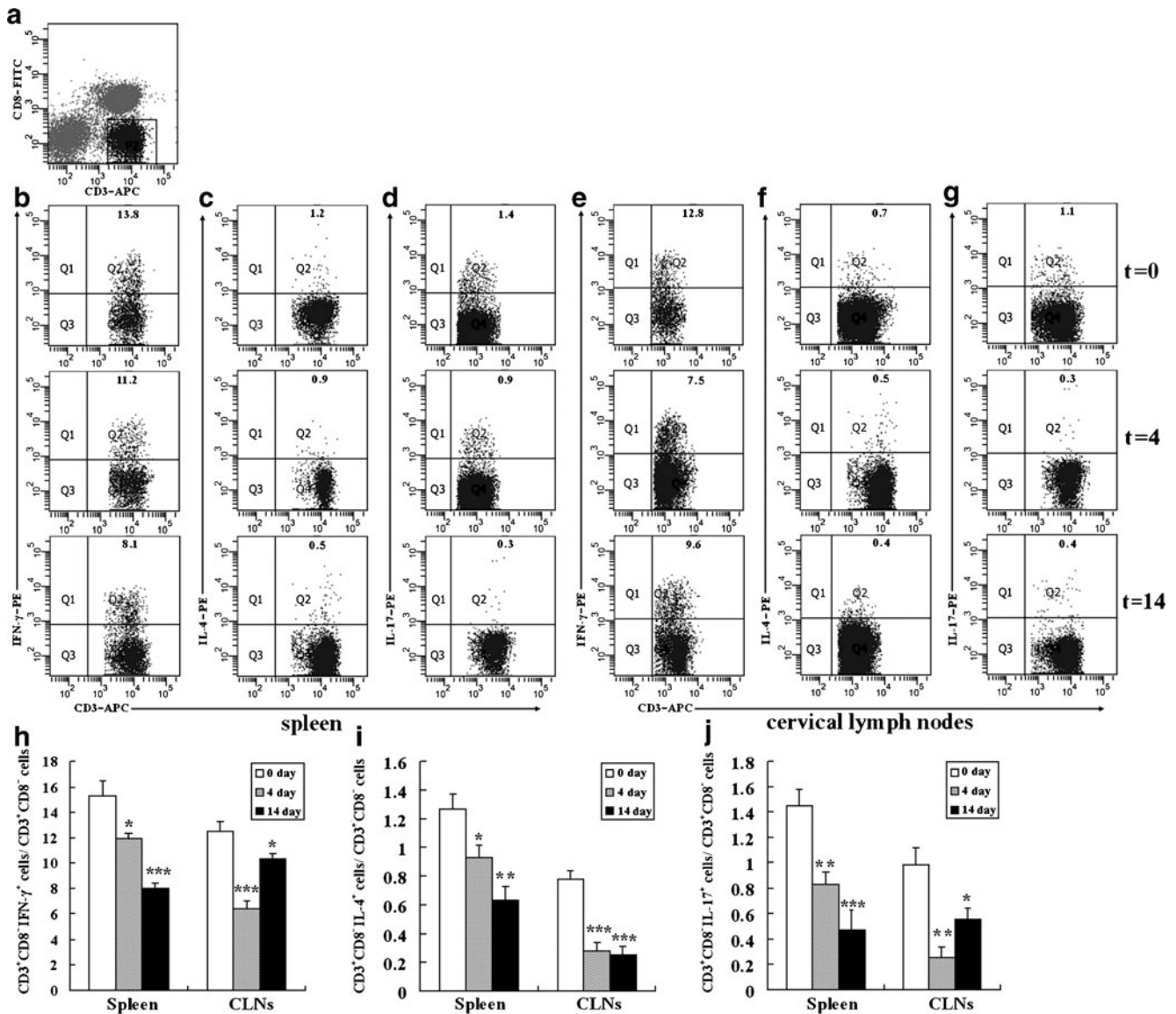
Fig. 6 Effects of nasal oxLDL on Th1, Th2, and Th17 cells in spleens and CLNs. ApoE$^{-/-}$ mice were nasally given with oxLDL daily for 5 days and were killed at days 4 and 14. Untreated mice were considered as a control. A, CD3$^+$CD8$^-$ Th cell subsets were gated. B through D, Representative results of CD3$^+$CD8$^-$IFN-γ$^+$ Th1 cells, CD3$^+$CD8$^-$IL-4$^+$ Th2 cells, and CD3$^+$CD8$^-$IL-17$^+$ Th17 cells in spleens estimated via FACS analysis, respectively. E through G, Representative results of CD3$^+$CD8$^-$IFN-γ$^+$ Th1 cells, CD3$^+$CD8$^-$IL-4$^+$ Th2 cells, and CD3$^+$CD8$^-$IL-17$^+$ Th17 cells in CLNs estimated by FACS analysis, respectively. H through J, The graphs represent the percentage of Th1, Th2, and Th17 cells in spleens and CLNs, respectively. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Data are mean ± SEM and are representative of at least three independent experiments

group vs oxLDL-treated group; 15.3±2.30 % vs 8.0±0.80 %, 1.27±0.20 % vs 0.63±0.20 %, 1.45±0.25 % vs 0.47±0.30 % in spleens, all $p<0.01$; 12.5±1.52 % vs 10.3±0.96 %, 0.78±0.12 % vs 0.25±0.12 %, 0.98±0.28 % vs 0.55±0.18 % in CLNs, all $p<0.05$) (Fig. 6).

Nasal oxLDL Induces TGF-β Production and Suppresses pro-inflammatory Cytokines Secretion in Splenocytes

To examine whether Tregs induced by nasal oxLDL affect the levels of cytokine produced by lymphocytes, we determined the cytokine secretion from splenocytes in response to stimulate with conA by ELISA. We analyzed the cytokine production from splenocytes on the 4th and 14th days after the last nasal treatment. Untreated mice were considered as a control. A marked increase in levels of TGF-β, but IL-10, was found in oxLDL-treated mice compared with untreated mice, respectively (Fig. 7A, all $p<0.01$). Moreover, we assessed the cytokine secretion from splenocytes at 16 and 24 weeks, and then found that TGF-β levels were also increased in group II compared with group I (Fig. 7C, $p<0.01$), in group IV and V compared with group III (Fig. 7D, all $p<0.05$), and in group

Fig. 7 Effects of nasal oxLDL on cytokines production in spleen cells. ApoE$^{-/-}$ mice were nasally administered with PBS or oxLDL 5 times, untreated or PBS-treated mice were considered as a control. A, C, and D, Splenocytes isolated from mice which were killed on 4th and 14th day after the last nasal treatment, 16 weeks, and 24 weeks of ages were stimulated with con A in vitro for 72 h, respectively. TGF-β production in supernatants was determined by ELISA. B, E, and F, Relative cytokine mRNA levels of IFN-γ, IL-4, IL-17, TGF-β, and IL-10 in unstimulated splenocytes collected from mice which were killed on 4th and 14th day after the last nasal treatment, 16 weeks, and 24 weeks of ages were measured by real time RT-PCR. Fold change relative to PBS-treated mice is shown. *$P<$0.05, **$P<$0.01, ***$P<$0.001 vs. untreated or PBS-treated mice. $^{\#}P<$0.05 vs. VI group. Data are mean ± SEM and are representative of at least three independent experiments
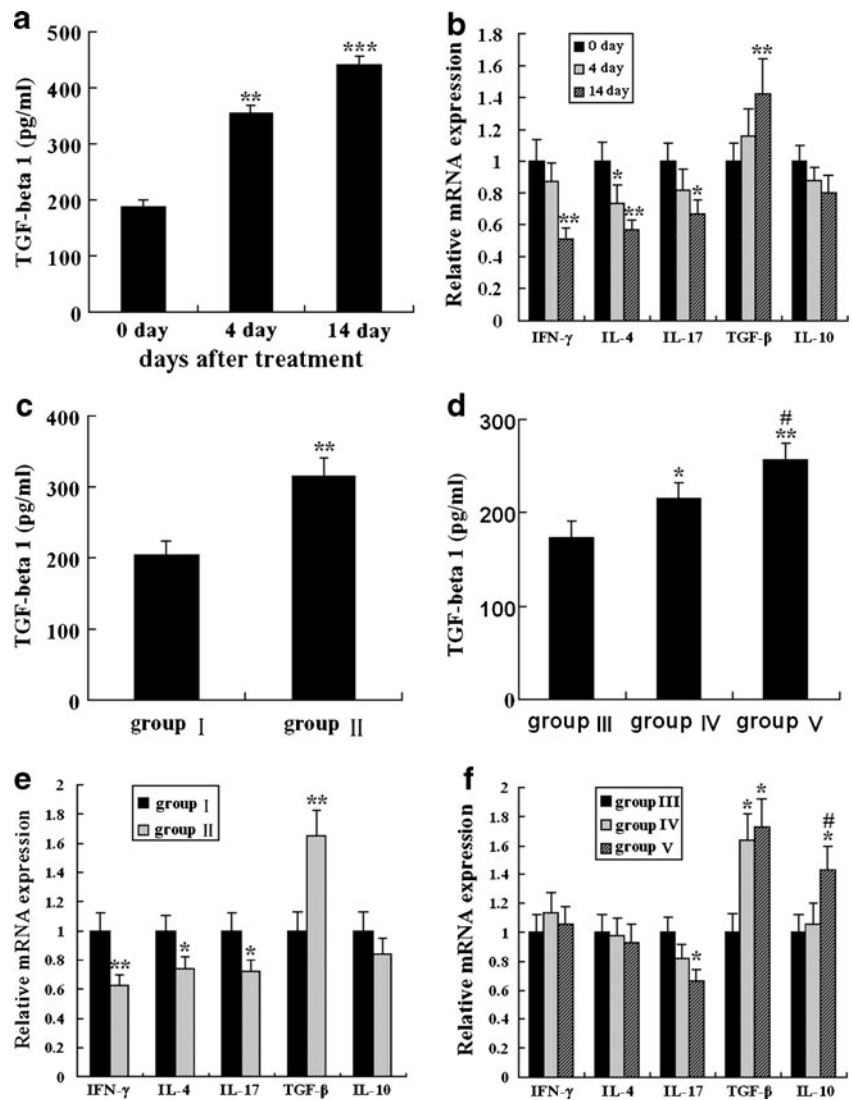

V compared with group IV (Fig. 7D, $p<0.05$). However, the levels of IL-10, IFN-γ, IL-4, and IL-17 were below the detection threshold in all experiments.

Thus, we estimated cytokine mRNA expression of unstimulated splenocytes via real-time RT-PCR. Intriguingly, oxLDL treatment resulted in an obvious decrease in mRNA levels of IL-4, and IL-17 on day 4 (Fig. 7B, all $p<0.05$) and in mRNA levels of IFN-γ, IL-4, and IL-17 on day 14 (Fig. 7B, all $p<0.01$). Importantly, a significant increase in mRNA TGF-β levels were found in oxLDL-treated mice on day 4 and 14 (Fig. 7B, all $p<0.05$), however, mRNA IL-10 levels were unchanged (all $p>0.05$). Similarly, increased TGF-β mRNA levels were observed in group II than in group I (Fig. 7E, $p<0.05$) and in group IV and V than in group III , respectively (Fig. 7F, all $p<0.05$), and decreased IL-17 mRNA levels were noticed in group II than in group I ($p<0.05$) and in group V than in group III ($p<0.05$). Additionally, down-regulated mRNA levels of IFN-γ and IL-4 were found in group II than in group I (all $p<0.05$),

however, mRNA levels of them were similar between group IV, V, and III (all $p>0.05$). Interestingly, the IL-10 mRNA levels were significantly increased in group V compared with group III, and IV (all $p<0.05$). Nevertheless, no differences were found in TGF-β mRNA levels between group IV and V ($p>0.05$) and in IL-10 mRNA levels between group III and IV ($p>0.05$).

Regulatory T Cells Markers and Inflammatory Markers in Atherosclerotic Plaques

To explore whether nasal oxLDL influences accumulation of Tregs and inflammatory cells in atherosclerotic plaques, we analyzed mRNA expression of TGF-β, CD25, Foxp3, IFN-γ, and adhesion molecules such as MCP-1 and VCAM-1 in the atherosclerotic lesions in the aortas. We found that mRNA expression of TGF-β and Foxp3 were significantly up-regulated in the atherosclerotic plaque in group II compared with group I
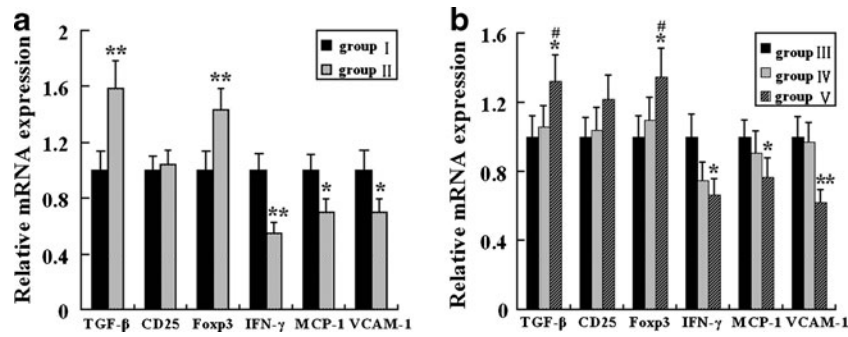
Fig. 8 Effects of nasal oxLDL on the relative mRNA expression of regulatory T cell markers and inflammatory markers in atherosclerotic plaques. A and B, Total RNA was extracted from aortas of 16-week-old and 24-week-old PBS- or oxLDL-treated mice. mRNA expression of regulatory T cell markers (TGF-β, CD25, Foxp3) and inflammatory markers (IFN-γ, MCP-1, VCAM-1) were quantitatively determined by real time RT-PCR. Fold change relative to PBS-treated mice is shown. *$P<0.05$, **$P<0.01$ vs. PBS-treated mice. #$P<0.05$ vs. VI group. Data are mean ± SEM and are representative of at least three independent experiments

(Fig. 8A, all $p<0.01$), and in group V compared with group III and IV (Fig. 8B, all $p<0.05$). However, there were no differences in mRNA expression of them between group IV and group III ($p>0.05$). Unexpectedly, the expression of CD25 mRNA was unchanged in group I and II (Fig. 8A, $p>0.05$), and in group III, IV, and V (Fig. 8B, $p>0.05$). Additionally, our data showed that mRNA expression of IFN-γ, MCP-1, and VCAM-1 were markedly reduced in group II compared to group I (Fig. 8A, all $p<0.05$) and in group V compared to group III (Fig. 8B, all $p<0.05$). Notably, IFN-γ mRNA expression was significantly reduced in group IV than in group III ($p<0.05$), and VCAM-1 mRNA expression was decreased in group V than in group IV ($p<0.05$). However, the expression of MCP-1, and VCAM-1 mRNA were no significant differences in between group IV and III (all $p>0.05$).

Nasal oxLDL Reduces the Proliferative Response of Splenocytes to oxLDL

OxLDL-specific T cells has been shown to be present in naive atherosclerosis prone mice [13]. To determine the effect of nasal oxLDL on the proliferation of oxLDL-specific T cells, we performed in vitro proliferation assays of splenocytes in response to oxLDL. 6-week-old mice were nasally treated with PBS or oxLDL as described above. Three days after the final nasal oxLDL administration, mice were immunized by intraperitoneal injection of 100 μg of oxLDL, and 2 weeks later splenocytes were isolated and co-cultured with 0, 1, 2.5, 5, 7.5 and 10 μg/mL of oxLDL, respectively. As shown in Fig. 9, stimulation with 1, 2.5, 5 and 7.5 μg/ml of oxLDL led to an increased proliferation of splenocytes in PBS-treated mice, whereas a concentration of oxLDL>10 μg/ml was shown to induce cytotoxicity.
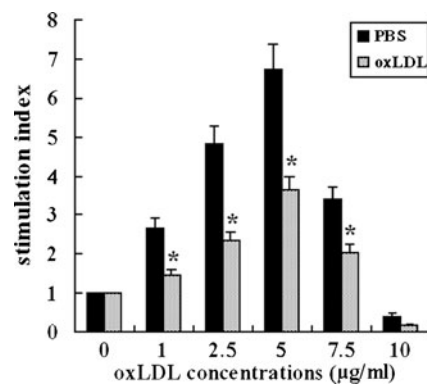
Notably, we observed a significant reduction in the proliferative response of splenocytes from oxLDL-treated mice to oxLDL compared with PBS-treated mice (all $p<0.05$).

The Atheroprotective Effect of Nasal oxLDL is Partly Depended on TGF-β

Our data indicated that TGF-β production was markedly increased by nasal application of oxLDL. Furthermore, increasing evidence showed that TGF-β plays an important role in the suppressive function of CD4+LAP+ and CD4+CD25+Foxp3+ Tregs [1, 3, 13, 14]. To investigate whether TGF-β is directly participated in inhibiting plaque formation after nasal oxLDL treatment, we conducted TGF-β neutralization study using an anti-TGF-β antibody in vivo. 6-week-old mice were nasally given 3 μg oxLDL daily for 5 consecutive days, and then were injected with 100 μg of neutralizing anti-TGF-β-Ab (group VI) or control

Fig. 9 Effects of nasal oxLDL on the proliferative response of spleen cells to oxLDL. Splenocytes isolated from PBS-treated and oxLDL-treated mice were restimulated in vitro with several different concentrations of oxLDL. *$P<0.05$. Data are mean ± SEM and are representative of at least three independent experiments

rat IgG (group VII) once a week from age 7 weeks to age 16 weeks. We observed a significant 28.8 % increase of atherosclerotic plaque formation in group VI compared to group VII (Fig. 10A, B, and C; 327,437.3±27,718.4 μm^2 vs 233,074.9±18,518.3 μm^2; $p<0.01$).

Discussion

Emerging experimental evidence has demonstrated the importance of oxLDL in the pathology of atherosclerosis. OxLDL and antibodies to oxLDL can be detected in atherosclerotic patients and experimental animals, which are shown the presence of atherosclerotic lesions [27, 28]. OxLDL presented by APCs is a potent pro-inflammatory chemoattractant for T lymphocytes, and results in effector T cells (in particular Th1 cells) activation [25]. These T cells respond to oxLDL, which can be found in plaque of atherosclerosis prone animals and humans using proliferation and cytokine secretion, promotes atherosclerosis [13, 29, 30]. Evidence from animal models showed that induction of neonatal tolerance

Fig. 10 Suppression of atherosclerosis is TGF-β dependent. A and B, Representative photomicrographs show Oil Red O and hematoxylin stained aortic root sections from group VI (n=8), group VII (n=8), respectively. C, Data from group VI and VII are shown: black circles represent animals from group VI, white circles represent animals from group VII

to oxLDL not only reduces T cells immune response, but also efficiently relieves atherosclerosis, suggesting that abrogating the T cells response to oxLDL can modify the course of atherosclerosis disease [29].

Mucosal administration of autoantigens can induce peripheral tolerance. Atherosclerosis-relevant autoantigens delivered via this route can silence Ag-specific T cells and suppress autoaggressive immune responses, and then attenuate the development of atherosclerosis [11–15]. In recent years, the classic routes for the generation of mucosal tolerance have mainly focused on the oral and nasal route. However, for practical reasons the nasal route might be lower in the dose of antigen used to achieve a similar effect, when compared with the oral route. Maron et al demonstrated that its effect on suppression of atherosclerosis was stronger in mice treated nasally 3 times every other day with 0.8 μg/dose Hsp65 than in mice treated orally 5 times every other day with 8 μg/dose Hsp65 [11], and moreover, Ma et al suggested that nasal acetylcholine receptor (AChR) administration, although only 1/1000 of the dose of oral AChR, is still as effective as oral application in preventing experimental autoimmune myasthenia gravis (EAMG), which is a classic autoimmune disease [31]. Recently, van Puijvelde et al reported that oral oxLDL (30 μg, 4 times) significantly attenuated atherosclerotic lesion formation [13]. In consideration of this, we decided to use a 3 μg/dose oxLDL for the treatment of mice by nasal administration.

In this study, we showed that nasal tolerance induction to oxLDL can inhibit the initiation and progression of atherosclerosis. This is in line with studies showing that nasal or oral tolerance induction to autoantigens [HSPs [11, 14], β$_2$-GPI [12], and oxLDL [13]] can attenuate the development of atherosclerosis, indicating that nasal oxLDL may be also a useful treatment approach for atherosclerosis. However, several previous studies have indicated that mucosal tolerance to autoantigens fails to suppress the advanced atherosclerosis [12, 14]. To address this issue, we carried out another investigation in the group V as described above, and then found that enhanced immunization once a week can lead to a significant reduction of plaque size in the progression of atherosclerosis. To our knowledge, this is the first study showing that appropriate enhanced immunization can contribute to inhibiting the advancement of the atherosclerotic lesion. This effect can be explained by the fact that up-regulation of TGF-β can suppress effector T cells immune response and attenuate the secretion of pro-inflammatory cytokines in the atherosclerotic plaques in mice enhanced immunization with oxLDL.

Several studies have certainly indicated that natural Tregs, CD4$^+$CD25$^+$Foxp3$^+$ Tregs, play a protective role in atherogenesis in mice [13, 32]. Our research team and
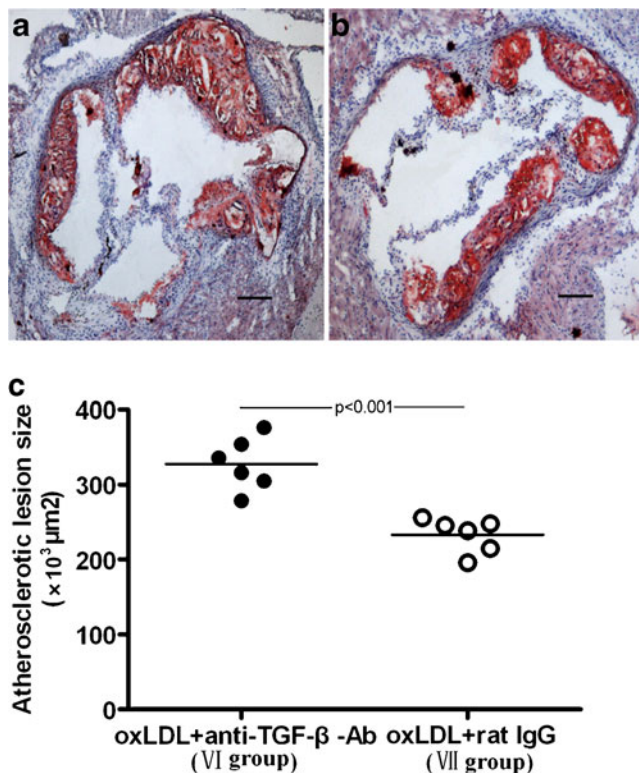
Mallat et al showed that other types of Tregs including Th3 or Tr1 cells may be responsible for the protective of atherosclerotic disease through the production of TGF-β or IL-10 [33, 34]. Recently, CD4$^+$LAP$^+$ Tregs, which secrete a large number of TGF-β or IL-10, have been attracting much attention as a new subset of Tregs in humans [1] and mice [2, 4–6]. Oida et al first identified a new regulatory CD4$^+$ T cell phenotype that is CD25$^-$ and LAP$^+$, and found that these cells can suppress CD4$^+$CD45RB^{high}-induced Colitis by a TGF-β-dependent mechanism [2]. Weiner's research team reported that oral anti-CD3-Ab induces CD4$^+$CD25$^-$LAP$^+$ Tregs that suppressed autoimmune encephalomyelitis (EAE), diabetes, systemic lupus erythematosus (SLE), and arthritis [4–6, 20]. These investigations suggested that CD4$^+$LAP$^+$ Tregs, which are induced by mucosal antigens, may be involved in immune modulation of autoimmune and inflammatory diseases. Although some investigator presumed that CD4$^+$LAP$^+$ Tregs that make TGF-β are probably Th3 cells, the relationship of these cells remains further scrutiny. In this study, we investigated the possible activation of Tregs. Four days after nasal oxLDL treatment, the number of CD4$^+$LAP$^+$ Tregs, particularly CD4$^+$CD25$^-$LAP$^+$ Tregs, was significantly increased in spleens and CLNs, moreover, the effect can last for 2 weeks at least. Additionally, we found a significant increase in CD4$^+$CD25$^+$Foxp3$^+$ Tregs in spleens and CLNs in oxLDL-treated mice. In contrast, a marked decrease in Th1, Th2, and Th17 cells in spleens and CLNs in oxLDL-treated mice was observed. These results indicate a shift from the T helper cells to CD4$^+$LAP$^+$ and CD4$^+$Foxp3$^+$ Tregs in preparation of spleens and CLNs after nasal oxLDL. Although the suppressive function of CD4$^+$CD25$^-$LAP$^+$ Tregs and CD4$^+$CD25$^+$ Tregs was unchanged, these results give us the reason for protecting against the initiation and progression of atherosclerosis by nasal oxLDL. This is consistent with a study by Sasski et al, who reported that mucosal application of anti-CD3-Ab attenuate atherosclerotic lesion formation by inducing an increase number of CD4$^+$LAP$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ Tregs and the elevated levels of TGF-β [3]. However, Kilngenberg et al found that intranasal immunization with apoB-100 can induce IL-10-producing Tr1 cells [35]. These studies demonstrated that the types of antigens used may account for these difference observed.

A number of studies have demonstrated that TGF-β plays an important role in inhibiting autoimmune and inflammatory diseases [3, 4, 36], and IL-17, produced by Th17 cells, can promote many autoimmune diseases [37]. TGF-β is able to suppress Th1 and Th2 immune responses [38], and inhibit the differentiation of CD4$^+$ T cells to Th17 cells in the presence of IL-6 [39]. We demonstrated that nasal application of oxLDL can increase TGF-β production from splenocytes. Although Th1 (IFN-γ), Th2 (IL-4), and Th17

(IL-17) cytokines production were undetected in the supernatants of splenocytes, a marked reduction of mRNA levels of IFN-γ, IL-4 and IL-17 was found in splenocytes in oxLDL-treated mice. Additionally, we found that nasal oxLDL can increase mRNA TGF-β and Foxp3 levels and decrease mRNA levels of IFN-γ, MCP-1, and VACM-1 in the lesions. Our results further confirmed that inflammatory cytokines aforementioned play a key role in atherogenesis.

Previous studies have demonstrated that anti-TGF-β-Ab treatment abrogate the suppression of Tregs in a mouse model of atherosclerosis [3] and colitis [40]. This finding could be further confirmed in our experiment. Surprisingly, we also found that enhanced immunization to oxLDL can increase the expression of IL-10 mRNA from splenoctyes besides TGF-β mRNA expression. Therefore, in spite of TGF-β plays a significant role in the suppressive function of CD4$^+$CD25$^-$LAP$^+$ Tregs, our data cannot exclude the possibility that IL-10 is also associated with the suppressive function of CD4$^+$CD25$^-$LAP$^+$ Tregs. In addition, nasal oxLDL reduced the proliferative response of splenoctyes to oxLDL, showing that a state of tolerance to oxLDL was established. Although this data did not allow us to rule out the possible beneficial effect of nasal tolerance induction on the humoral immune response because of no detection of oxLDL-specific antibody levels in this study, we suggest that nasal tolerance induction to oxLDL suppresses atherosclerosis by the enhanced cellular immune response.

Taken together, we demonstrated that nasal oxLDL treatment suppresses atherosclerosis development via inducing CD4$^+$CD25$^+$Foxp3$^+$ and CD4$^+$LAP$^+$ Tregs, which secrete a large number of TGF-β. Although the mechanism by which nasal administration of oxLDL increases Tregs remains to be investigated, these results are able to validate the fact that nasal tolerance induction to autoantigens may be an effective means for anti-atherosclerosis.

References

1. Gandhi R, Farez MF, Wang Y, Kozoriz D, Quintana FJ, Weiner HL. Cutting Edge: Human Latency-Associated Peptide$^+$ T Cells: A Novel Regulatory T Cell Subset. J Immunol. 2010;184:4620–4.
2. Oida T, Zhang X, Goto M, Hachimura S, Totsuka M, Kaminogawa S, Weiner HL. CD4$^+$CD25$^-$ T cells that express latency-associated peptide on the surface suppress CD4$^+$CD45RB^{high}-induced colitis by a TGF-beta-dependent mechanism. J Immunol. 2003;170:2516–22.

3. Sasaki N, Yamashita T, Takeda M, Shinohara M, Nakajima K, Tawa H, Usui T, Hirata K. Oral anti-CD3 antibody treatment induces regulatory T cells and inhibits the development of atherosclerosis in mice. Circulation. 2009;120:1996–2005.

4. Ochi H, Abraham M, Ishikawa H, Frenkel D, Yang K, Basso AS, Wu H, Chen ML, Gandhi R, Miller A, Maron R, Weiner HL. Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4$^+$CD25$^-$LAP$^+$ T cells. Nat Med. 2006;12:627–35.

5. Ishikawa H, Ochi H, Chen ML, Frenkel D, Maron R, Weiner HL. Inhibition of autoimmune diabetes by oral administration of anti-CD3 monoclonal antibody. Diabetes. 2007;56:2103–9.

6. Wu HY, Center EM, Tsokos GC, Weiner HL. Suppression of murine SLE by oral anti-CD3: inducible CD4$^+$CD25$^-$LAP$^+$ regulatory T cells control the expansion of IL-17$^+$ follicular helper T cells. Lupus. 2009;18:586–96.

7. Ross R. Atherosclerosis–an inflammatory disease. N Engl J Med. 1999;340:115–26.

8. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. Nat Rev Immunol. 2006;6:508–19.

9. Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. Circulation. 2007;116:1832–44.

10. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, Witztum JL. Innate and acquired immunity in atherogenesis. Na Med. 2002;8:1218–26.

11. Maron R, Sukhova G, Faria AM, Hoffmann E, Mach F, Libby P, Weiner HL. Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. Circulation. 2002;106:1708–15.

12. George J, Yacov N, Breitbart E, Bangio L, Shaish A, Gilburd B, Shoenfeld Y, Harats D. Suppression of early atherosclerosis in LDL-receptor deficient mice by oral tolerance with beta 2-glycoprotein I. Cardiovas Res. 2004;62:603–9.

13. van Puijvelde GH, Hauer AD, de Vos P, van den Heuvel R, van Herwijnen MJ, van der Zee R, van Eden W, van Berkel TJ, Kuiper J. Induction of oral tolerance to oxidized low-density lipoprotein ameliorates atherosclerosis. Circulation. 2006;114:1968–76.

14. van Puijvelde GH, van Es T, van Wanrooij EJ, Habets KL, de Vos P, van der Zee R, van Eden W, van Berkel TJ, Kuiper J. Induction of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis. Arterioscler Thromb Vasc Biol. 2007;27:2677–83.

15. Xiong Q, Li J, Jin L, Liu J, Li T. Nasal immunization with heat shock protein 65 attenuates atherosclerosis and reduces serum lipids in cholesterol-fed wild-type rabbits probably through different mechanisms. Immunol Lett. 2009;125:40–5.

16. Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A, Forsthuber T, Lehmann PV, Kaufman DL. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J Exp Med. 1996;183:1561–7.

17. Garcia G, Komagata Y, Slavin AJ, Maron R, Weiner HL. Suppression of collagen-induced arthritis by oral or nasal administration of type-II collagen. J Autoimmun. 1999;13:315–24.

18. Nussenblatt R. Orally and nasally induced tolerance studies in ocular inflammatory disease: guidance for future interventions. Ann N Y Acad Sci. 2004;1029:278–85.

19. Broere F, Wieten L, Klein Koerkamp EI, van Roon JA, Guichelaar T, Lafeber FP, van Eden W. Oral or nasal antigen induces regulatory T cells that suppress arthritis and proliferation of arthritogenic T cells in joint draining lymph nodes. J Immunol. 2008;181:899–906.

20. Wu HY, Maron R, Tukpah AM, Weiner HL. Mucosal anti-CD3 monoclonal antibody attenuates collagen-induced arthritis that is associated with induction of LAP$^+$ regulatory T cells and is enhanced by administration of an emulsome-based Th2-skewing adjuvant. J Immunol. 2010;185:3401–7.

21. Redgrave TG, Roberts DC, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal Biochem. 1975;65:42–9.

22. Holven KB, Aukrust P, Holm T, Ose L, Nenseter MS. Folic acid treatment reduces chemokine release from peripheral blood mononuclear cells in hyperhomocysteinemic subjects. Arterioscler Thromb Vasc Biol. 2002;22:699–703.

23. Wolvers DA, Coenen-de Roo CJ, Mebius RE, van der Cammen MJ, Tirion F, Miltenburg AM, Kraal G. Intranasal induced immunological tolerance is determined by characteristics of the draining lymph nodes: studies with OVA and human cartilage gp-39. J Immunol. 1999;162:1994–8.

24. Cheng X, Chen Y, Xie JJ, Yao R, Yu X, Liao MY, Ding YJ, Tang TT, Liao YH, Cheng Y. Suppressive oligodeoxynucleotides inhibit atherosclerosis in ApoE(−/−) mice through modulation of Th1/Th2 balance. J Mol Cell Cardiol. 2008;45:168–75.

25. Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA, Witztum JL. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J Exp Med. 2004;200:1359–70.

26. Tang H, Zhong Y, Zhu Y, Zhao F, Cui X, Wang Z. Low responder T cell susceptibility to the suppressive function of regulatory T cells in patients with dilated cardiomyopathy. Heart. 2010;96:765–71.

27. Salonen JT, Ylä-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, Nyyssänen K, Palinski W, Witztum JL. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. Lancet. 1992;339:883–7.

28. Ylä-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D, Witztum JL. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. Arterioscler Thromb. 1994;14:32–40.

29. Stemme S, Faber B, Holm J, Wiklund O, Witztum JL, Hansson GK. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. Proc Natl Acad Sci USA. 1995;92:3893–7.

30. Mallat Z, Taleb S, Ait-Oufella H, Tedgui A. The role of adaptive T cell immunity in atherosclerosis. J Lipi Res. 2009;50:S364–9.

31. Ma CG, Zhang GX, Xiao BG, Link J, Olsson T, Link H. Suppression of experimental autoimmune myasthenia gravis by nasal administration of acetylcholine receptor. J Neuroimmunol. 1995;58:51–60.

32. Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hasson GK, Klatzmann D, Tedgui A, Mallat Z. Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med. 2006;12:178–80.

33. Ji QW, Guo M, Zheng JS, Mao XB, Peng YD, Li SN, Liang ZS, Dai ZY, Mao Y, Zeng QT. Downregulation of T helper cell type 3 in patients with acute coronary syndrome. Arch Med Res. 2009;40:285–93.

34. Mallat Z, Gojova A, Brun V, Esposito B, Fournier N, Cottrez F, Tedgui A, Groux H. Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apoE$^{-/-}$ mice. Circulation. 2003;108:1232–7.

35. Klingenberg R, Lebens M, Hermansson A, Fredrikson GN, Strodthoff D, Rudling M, Ketelhuth DF, Gerdes N, Holmgren J, Nilsson J, Hansson GK. Intranasal immunization with an ApoB-100 fusion protein induces antigen-specific regulatory T cells and reduces atherosclerosis. Arterioscler Thromb Vasc Biol. 2010;30:946–52.

36. Chen ML, Yan BS, Bando Y, Kuchroo VK, Weiner HL. Latency-Associated Peptide Identifies a Novel CD4+CD25+Regulatory T Cell Subset with TGFβ-Mediated Function and Enhanced

Suppression of Experimental Autoimmune Encephalomyelitis. J Immunol. 2008;180:7327–37.

37. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005;6:1133–41.

38. Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK. Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. J Clin Invest. 2003;112:1342–50.

39. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441:235–8.

40. Powrie F, Carlino J, Leach MW, Mauze S, Coffman RL. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J Exp Med. 1996;183:2669–74.