Involvement of lipid peroxidation and Th17/Th1/Treg imbalance in *Aggregatibacter actinomycetemcomitans*-accelerated atherosclerosis

Ru Jia

Microbiology and Immunology
Nihon University School of Dentistry at Matsudo

Director: Prof. Masafumi Yamamoto
Prof. Koh Shibutani

Contents

1. Preface

This article is constructed with a main reference paper "Periodontal Pathogen Accelerates Lipid Peroxidation and Atherosclerosis" in Journal of Dental Research, and a reference paper "Functional Imbalance between Th17, Th1, and Treg Cells in *A. actinomyctemcomitans* -Accelerated Atherosclerosis" in International Journal of Oral-Medical Sciences.

2. Abstract

Recent studies have shown an association between periodontal disease and cardiovascular disease. We previously reported that *Aggregatibacter actinomycetemcomitans* (*Aa*) bacteremia accelerated atherosclerosis in apolipoprotein E-deficient spontaneously hyperlipidemic (Apoe[shl]) mice. In this study, we investigated whether live cells were necessary for this process or whether lipopolysaccharide (LPS) alone is sufficient to cause an increase in atherosclerotic damage. Mice were treated intravenously with live *Aa* HK1651, heat-killed (H.K.) *Aa*, or *Aa* LPS three times a week for 3 weeks and killed at 15 weeks of age. The areas of the aortic sinus that were covered with atherosclerotic plaques were significantly larger in mice treated with live *Aa*, H.K. *Aa*, or *Aa* LPS compared with vehicle-challenged mice. The order of the extent of atherosclerosis was live *Aa* > H.K. *Aa* > *Aa* LPS > sham. Toll and NOD-like receptor mRNA expression significantly increased in the live *Aa*, H.K. *Aa*, and *Aa* LPS treatment groups. *Aa* challenge markedly promoted the oxidation of LDL through oxidative stress involving NADPH oxidase- and myeloperoxidase-derived reactive oxygen species. These results suggest that *Aa* promotes innate immune signaling and the oxidation of LDL, and may thus facilitate atheroma development. In the next study, we investigated whether the functional imbalance between Th17, Th1, and regulatory T (Treg) cells, existed in *Aa*-challenged Apoe[shl] mice. The mice were intravenously treated with live *Aa* HK1651 or vehicles. Histomorphometric features of atheromatous lesions, IL-17^{+}CD4^{+}, IFN-γ^{+}CD4^{+}, and Foxp3^{+}CD4^{+} cell frequencies, serum IL-17, IL-6, TGF-β, IFN-γ , and IL-10 levels, and gene expression of Th17-related molecules were examined. *Aa* challenge induced a Th17/Th1 shift in Apoe[shl] mice. *Aa*-challenged splenic Th1 and Th17 cells greatly increased in contrast with reduction in Treg cells after their transient increase at 13 weeks. Serum cytokine levels of IL-6 were significantly enhanced during *Aa*-challenge. Similarly, gene expression of differentiation factors (IL-6, IL-17RA and IL-21), growth/stabilization factor (IL-23), and transcription factor (STAT3) involved in the development of Th17 cells, as well

as Th1-related IFN-γ were also stimulated in *Aa*-challenged mice. These results suggest that Th17/Th1/Treg imbalance affect the progression of *Aa*-accelerated atherosclerosis.

3. Introduction

We previously showed that *Aggregatibacter actinomyctemcomitans* (*Aa*) and *Porphyromonas gingivalis* accelerate the progression of atherosclerosis in BALB/c apolipoprotein E (apoE)-deficient spontaneously hyperlipidemic (C. KOR-Apoe[shl]) mice [1, 2]. Furthermore, previous observations suggested that inflammation caused by periodontopathic bacteria may play a synergistic role with other pre-existing factors, such as hyperlipidemia, resulting in the development of atherosclerosis [3]. These observations support the hypothesis that a periodontal pathogen is not an independent risk factor, but acts in concert with hyperlipidemia to exacerbate atherosclerosis lesion formation. However, whether live *Aa* was necessary for this process or whether its component alone is sufficient to cause an increase in atherosclerotic damage remains unclear. Periodontal bacteria such as *Aa* and their components may have direct access to the circulation through bleeding periodontal pockets. *Aa* has been detected in human atherosclerotic plaques [4-6]. Furthermore, patients with periodontitis are known to suffer from endotoxemia, which is a risk factor for cardiovascular disease [7]. *Aa*-derived lipopolysaccharide (LPS) complexed with low-density lipoprotein (LDL) may enter the vessel wall, activating inflammatory cells to produce MMP-9 [8]. LPS isolated from *Aa* is known to transform macrophages into lipid-laden foam cells [9]. The pro-inflammatory response induced by *Aa* leukotoxin is also associated with the pathogenesis of atherosclerosis and myocardial infarction [10, 11].

Atherosclerosis is a chronic inflammatory disease in which lipoproteins accumulate, eliciting an inflammatory response in the arterial wall. An important consequence of this process is the cellular oxidation of LDL, which converts the lipoprotein to a highly atherogenic form [12]. Oxidative stress induced by reactive oxygen species (ROS) *via* NADPH oxidase and myeloperoxidase (MPO) is responsible for LDL oxidation and impairment of cellular function. Moreover, recent studies have shown that innate immune signaling in aortic tissue may be associated with atherogenesis [13]. The present study was performed to examine whether

systemic circulatory challenge with live *Aa*, heat-killed *Aa*, or *Aa* LPS promotes and accelerates the development of atherosclerotic lesions in C. KOR-Apoe[shl] mice as an alternative model of *apo*E deficiency [1]. Furthermore, we assessed the involvement of innate immune signaling molecules such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), ox-LDL, other oxidative stress biomarkers, and ROS-generating oxidase in *Aa*-accelerated atherosclerosis.

It is generally accepted that the adaptive and innate immune systems are involved in every phase of the atherosclerotic process. Among the immune cells that accumulate in atherosclerotic lesion [14, 15], CD4^{+} T cells perform important duties during the development of the plaque. When these cells infiltrate into the plaques, they can secrete a number of inflammatory cytokines and participate in the various phases of immune and inflammatory responses in the pathogenesis of atherosclerosis. The up-regulation of T helper cell 1 (Th1) response has been found in the local atherosclerotic lesions and circulating lymphocytes in atherosclerotic animal models and patients with acute coronary syndrome, suggesting that Th1/Th2 imbalance plays an important role in the development of atherosclerosis and plaque rupture [16, 17]. Furthermore, recent study demonstrated that the peripheral Th17/Treg imbalance also existed in patients with acute coronary syndrome, suggesting a potential role of Th17/Treg imbalance in plaque destabilization [18]. De Boer et al. also reported that the frequency of naturally occurring Treg cells decreased in all developmental stages of human atherosclerotic lesions [19]. Th17 cells have been reported as a new subpopulation of CD4^{+} T cells [20], and their key regulator for Th17-cell lineage differentiation is the retinoic acid-related orphan nuclear receptor gamma t (RORγt) [21]. Th17 cells produce the signature cytokine interleukin-17 (IL-17) and participate in the induction of inflammation [22, 23]. IL-17 and Th17 cells have been implicated in the pathogenesis of many chronic infectious disease caused by microbial pathogens [24, 25]. The increase of Th17 cells in chronic periodontal patients and by *P. gingivalis* exposure to peripheral blood mononuclear cells in vitro were also recognized [26, 27].

We have previously shown that *Aa* bacteremia accelerated the progression of atherosclerosis through induction of inflammation and promotion of lipid oxidation in C. KOR-Apoe[shl] mice [2, 28]. Periodontal bacteria such as *Aa* and their components may have direct access to the circulation through bleeding periodontal pockets. *Aa* has been detected in human atherosclerotic plaques [4, 29, 6]. However, little is known about how *Aa* influences T cell function in progression of atherosclerosis. Therefore, in this study, we investigated Th1/Th17/Treg distribution at different levels including cell frequencies in spleen, related serum cytokine secretion and related molecule-specific mRNA expression in spleen of Apoe[shl] mice.

4. Materials and Methods

Bacterial Strains and LPS

Aa HK1651 (ATCC 700685) was cultured anaerobically as described previously [2]. Bacteria were harvested from Todd-Hewitt broth (BBL, Cockeysville, MD, USA) supplemented with 1% yeast extract (Difco Laboratories, Detroit, MI, USA) and re-suspended in phosphate-buffered saline (PBS) for intravenous (i.v.) infection. H.K. Aa was prepared by being heated at 100°C for 30 min. Aa LPS was prepared from Aa HK1651 by the hot phenol-water method, and was purified by enzymatic hydrolysis with nuclease and protease [30].

Mice

Ten-week-old female (C. KOR-Apoe^{shl}) mice [31] obtained from Japan SLC Inc. (Hamamatsu, Japan) were provided a regular chow diet and water *ad libitum*. The Institutional Animal Care and Use Committee of Nihon University approved all the animal protocols. After 1 wk, the mice were randomly divided into 4 groups (n = 6 *per* group). The mice were injected i.v. with 0.1 mL of live Aa (10^8 CFU/mouse), H.K. Aa (10^8 CFU/mouse), Aa LPS (50 μg/mouse), or PBS 3 times a wk for 3 wks as described previously [2]. Mice were sacrificed 1 wk after the last injection. Blood samples were collected in heparinized syringes from the orbital veins of mice anesthetized with Isozol (Nichi Iko, Toyama, Japan).

In separate experiment, 11-week-old female mice were randomly divided into 8 groups, and used for experiments. The mice were treated i.v. with 0.1 mL of live Aa (10^8 CFU/mouse) or PBS three times a week for 1, 2, 3 weeks. Mice were killed at 12, 13, 14 and 15 weeks (n = 6 per group) after injection. Blood samples were collected in heparinized syringes from the orbital veins of mice anesthetized with Isozol (Nichi Iko, Toyama, Japan).

Quantification of atherosclerotic lesion area

The heart and aortic tree were perfused through the left ventricle with ice-cold 0.9% saline for 10 min. The heart was then carefully dissected and removed. The upper half of the heart, which contains the aortic origin, was separated and embedded in Tissue-Tek (Fisher Scientific, Newark, DE, USA) OCT compound in cryomolds, and frozen sections of the proximal aorta were prepared as described previously [2]. Briefly, 6-μm-thick sections pre mouse were stained with Oil Red O to visualize neutral lipid and counterstained with hematoxylin. Total lesion area per slide and percentage of the aortic lumen occupied by lesions per section were calculated using image analysis software (Lumina Vision, Mitani Co., Fukui, Japan). The values of 15 sections per animal were averaged and expressed as the mean lesion area and percentage of the lumen of the proximal aorta occupied by lesions per section per animal.

Analysis of Gene Expression by Quantitative Real-Time RT-PCR

Total RNA purified from the aorta (n = 6 *per* group) was reverse-transcribed with oligo(dT) primers using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to generate cDNA. Quantitative real-time RT-PCR analyses were performed in a Thermal Cycler Dice real-time PCR system (Takara, Shiga, Japan) as described previously [2]. The primers are shown in the Table 1. PCR consisted of an initial denaturation step (95°C for 10 min), followed by 40 cycles at 95°C for 15 sec, 55°C for 25 sec, and 60°C for 35 sec. Each gene was tested in triplicate. For receptors for advanced glycation end-products (RAGE) and inducible nitric oxide synthase (iNOS), PCR reactions were performed with the following cycle conditions: 95°C for 10 min, followed by 40 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. Target RNA levels were normalized to that of GAPDH mRNA.

Immunohistochemistry

Mouse aortas were fixed with 10% formalin and embedded in paraffin [32]. Paraffin-embedded sections of aortic sinus (4 μm thick) were then incubated with anti-mouse-4-hydroxynonenal (4-HNE) monoclonal antibody (Ab) (Nichirei Bioscience, Tokyo, Japan), HOCL-oxidized LDL (ox-LDL) polyclonal Ab (Nikken Seil Co., Ltd.), and phospholipase A2 (PLA2) polyclonal Ab (ab58375; Abcam, Cambridge, UK), followed by N-Histofine Simple Stain MAX PO (G) (Nichirei Bioscience) and diaminobenzidine substrate (DAKO Japan, Tokyo, Japan), according to the manufacturers' instructions. Nuclei were counterstained with hematoxylin.

Serum Analysis

Sera were obtained prior to the animals' death as described previously [2]. Serum was isolated from blood by centrifugation at 2500 g for 20 min after clotting at room temperature. The level of ox-LDL was determined using a Mouse Oxidized Low-density Lipoprotein enzyme-linked immunosorbent assay (ELISA) Kit (CUSABIO Biotech CO., Ltd., Newark, DE, USA). Serum 8-hydroxy-2'-deoxy-guanosine (8-OHdG) and MPO levels were respectively determined using a Highly Sensitive 8-OHdG Check ELISA Kit and an MPO ELISA Kit (Nikken Seil Co., Ltd., Shizuoka, Japan).

The concentration of IL-17, IL-6, transforming growth factor- (TGF-β), interferon-γ (IFN-γ), and IL-10 in serum was measured by ELISA following the manufacture's instructions (ELISA kits for IL-17 and TGF-β, R&D Systems,Minneapolis, MN; ELISA kits for IFN-γ, IL-6 and IL-10, Thermo Scientific, Rockford, IL).

Flowcytometric analysis of Th17, Th1, and Treg cells

Single-cell suspensions were obtained from the spleen of mice treated with *Aa* or PBS for 1, 2 and 3 weeks. For intracellular staining, the cell suspensions were

stimulated for 5 h with 50 ng/ml phorbol 12-myristate 13-acetate (PMA: Sigma-Aldrich, St. Louis, MO, USA), 1mM ionomycin (Sigma-Aldrich) and 500 ng/ml monensin (Sigma-Aldrich). For surface staining of cells, a fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 monoclonal antibody (Becton Dickingson) was used. After surface staining, fixation and permeabilization, cells were stained with phycoerythrin (PE) anti-mouse-IL-17 for Th17 detection, PE anti-mouse IFN-γ for Th1 detection, or PE anti-mouse fork head/singed helix transcription factor (Foxp3) for Treg cell detection according to the manufacturer's instructions (all of the Abs were from eBioscience, San Diego, CA). Isotype controls were given to enable correct compensation and confirm antibody specificity. Stained cells were analyzed by flow cytometric analysis using a FACS calibur cytometer equipped with CellQuest software (BD Bioscience Pharmingen).

Statistical Analysis

The data are presented as the mean ± the standard deviation (SD). Differences in total atherosclerotic plaque accumulation were assessed by one-way ANOVA followed by the Tukey-Kramer multiple comparison test. A P value of <0.05 was considered statistically significant.

5. Results

Atherosclerotic Plaque Formation in Aortic Sinuses

Histomorphometric analysis revealed a significant increase in atherosclerotic plaque accumulation in live *Aa*-, H.K. *Aa*-, and *Aa* LPS-challenged mice compared with sham-injected mice, with the percentage of the total lumen of the proximal aorta occupied by lesions showing the same pattern (Fig. 1A, 1C: $7093 \pm 2644 \ \mu m^2$ for *Aa*-challenged mice, $5678 \pm 1265 \ \mu m^2$ for H.K. *Aa*-challenged mice, and $4149 \pm 1630 \ \mu m^2$ for *Aa* LPS-challenged mice *vs.* $91 \pm 91 \ \mu m^2$ for sham-injected mice; $p < 0.01$) (Fig. 1B, 1C: $4.04 \pm 1.09\%$ for *Aa*-challenged mice, $3.72 \pm 0.40\%$ for H.K. *Aa*-challenged mice, and $2.28 \pm 0.70\%$ for *Aa* LPS-challenged mice *vs.* 1.14 ± 0.24 for sham-injected mice; $p < 0.01$). The order of extent of atherosclerosis was live *Aa* > H.K. *Aa* > *Aa* LPS > PBS. There was a significant difference between *Aa*-challenged mice and *Aa* LPS-challenged mice ($p < 0.05$).

Aa Challenge Up-regulates TLR and NLR Expression

Real-time RT-PCR analysis showed that live *Aa* infection significantly ($p < 0.05$) enhanced the expression TLR-2-, TLR-4-, TLR-9-, and NOD-1-specific mRNA in the aorta, while H.K. *Aa* and *Aa* LPS significantly ($p < 0.05$) increased TLR-4-, NOD-1-, and NOD-2-specific mRNA levels in the aorta (Fig. 2). In terms of TLR-2 and TLR-9 mRNA expression, there was a significant difference between *Aa*-challenged mice and H.K. *Aa*-challenged mice as well as *Aa*-challenged mice and *Aa* LPS-challenged mice ($p < 0.05$). With regard to TLR-4 mRNA expression, there was a significant difference between *Aa*-challenged mice and *Aa* LPS-challenged mice ($p < 0.05$). For NOD1 mRNA expression, there was a significant difference between *Aa*-challenged mice and H.K. *Aa*-challenged mice ($p < 0.05$), and for NOD2 mRNA expression, there was a significant difference between *Aa*-challenged and *Aa* LPS-challenged mice.

Immunohistochemical Analysis of 4HNE, PLA$_2$, and ox-LDL

We evaluated the effect of *Aa* on lipid peroxidation in the aorta. To determine whether oxidative changes occurred in the aortic in Apoe^{sh1} mice, we stained cryosections of the aortic arch immunohistochemically using 4HNE, ox-LDL, and PLA2 Abs. Immunostaining of the paraffin-embedded aortic sinus sections revealed marked up-regulation of 4HNE production in *Aa*-, H.K. *Aa*-, and *Aa* LPS-challenged mice compared with the controls (Fig. 3). We observed a positive ox-LDL area in the aortic sinuses of mice stimulated with *Aa* and (to a lesser extent) H.K. *Aa* and *Aa* LPS (Fig. 3). PLA2 was also detected immunohistochemically in cryosections of aortic sinus from *Aa*- and (to a lesser extent) H.K. *Aa*- and *Aa* LPS-challenged mice (Fig. 3).

Serum ox-LDL, 8-OHdG, and MPO Levels

Serum ox-LDL levels were increased in *Aa*-challenged mice compared with H.K. *Aa*-challenged, *Aa* LPS-challenged, and sham-injected mice, although the difference was not statistically significant (Fig. 4A: 1.25 ± 0.39 μmol/mL for *Aa vs.* 0.62 ± 0.23 μmol/mL for H.K. *Aa*, 0.60 ± 0.46 μmol/mL for *Aa* LPS, and 0.55 ± 0.55 mol/mL for PBS). Furthermore, serum 8-OHdG and MPO levels were significantly increased in the *Aa*-challenged group compared with those in the H.K. *Aa*-challenged, *Aa* LPS-challenged, and sham-injected groups (Fig. 4A: 8-OHdG, 1.55 ± 0.02 ng/mL for *Aa vs.* 1.45 ± 0.13 ng/mL for H.K. *Aa*, 1.48 ± 0.61 ng/mL for *Aa* LPS, and 1.30 ± 0.03 ng/mL for PBS, $p < 0.05$; MPO, 8.25 ± 0.35 ng/mL for *Aa vs.* 7.35 ± 1.53 ng/mL for H.K. *Aa*, 6.77 ± 0.75 ng/mL for *Aa* LPS, and 6.1 ± 0.14 ng/mL for PBS, $p < 0.05$).

NADPH Oxidase Expression in the Aorta

Aa and *Aa*-LPS challenge induced a significant increase in the mRNA expression of the NADPH oxidase-related genes NOX-2 ($p < 0.05$) and NOX-4 ($p < 0.05$) compared with the H.K. *Aa*-challenged and PBS-injected groups (Fig. 4B).

NOX-1 mRNA levels were also slightly increased in the *Aa*-challenged group ($p > 0.05$; Fig. 4B).

Expression of Cav-1, RAGE, and iNOS in the Aorta

To further analyze the influence of oxidative stress in infected animals, we measured aorta expression of Caveolin-1 (Cav-1), RAGE, and iNOS. Mice inoculated with *Aa* displayed a significant increase in the mRNA expression of RAGE ($p < 0.05$) and Cav-1 ($p < 0.05$) compared with the PBS-treated group (Fig. 4C). Mice inoculated with H.K. *Aa* also showed a significant increase in the Cav-1 mRNA level ($p < 0.05$) compared with the sham-injected group (Fig. 4C).

Flow cytometry analysis of Th17/Th1/Treg balance in splenic cells

The levels of splenic $CD4^+$ $IL-17^+$ cells were significantly higher in *Aa*-challenged mice than those in their wild-type littermates at all time points, moreover it achieved the maximal value at 14 weeks time dependently (Fig. 5). Consistent with $IL-17^+$ cells, the levels of $CD4^+$ $IFN\text{-}\gamma^+$ cells in the spleen of *Aa*-challenged mice were also higher compared with age-matched control, and achieved the maximal value at 14 weeks. Although the levels of $CD4^+$ $Foxp3^+$ cells were also reached the maximum at 13 weeks, they obviously decreased at 14 weeks. This finding suggests that *Aa*-challenge time-dependently increases splenic Th1 and Th17 cell populations in contrast with reduction in Treg cells after their transient increase at 13 weeks.

Serum IL-17, IFN-γ, IL-6, IL-10 and TGF-β levels

Aa-challenge significantly increased serum IL-6 level (Fig. 6: 206.35±8.84 pg/ml vs. 104.15±5.87 $p<0.05$) compared with PBS inoculated group. IL-17, IFN-γ and IL-10 levels were also higher in *Aa*-challenged mice than those in contol group.(Fig. 6: IL-17, 1861.67±890.15 pg/mL vs.1248±530.42 pg/mL,

IFN-γ,1300.33±458.12 pg/mL vs.1075.56±230.1 pg/mL, IL-10, 312.67 ±105.2 pg/mL vs.206.23±89.34 pg/mL), although this did not reach statistical significance. Furthermore, we observed no significant difference in the production of TGF-β.

Th17, Th1 and Treg related molecules-specific mRNA expression in spleen

To examine the involvement of Th17, Th1, and Treg-related molecules in atherosclerosis enhanced by *Aa*-challenge, we analyzed the kinetics of their mRNA expression in the spleen of mice at 15 weeks by real-time RT-PCR.   The analysis showed that *Aa*-challenge induced a significant increase in IL-6, IL-17RA, IL-21, IL-23 and IFN-γ mRNA levels ($p<0.05$) and slight increase in TGF-β, STAT3, STAT4 and Foxp3 mRNA levels in spleen (Fig. 7). However, there was no significant difference in the expression of T-bet-specific mRNA between *Aa*-challenged and control mice.

6. Discussion

Intravenous injection may not be a physiologically relevant model for the study of *Aa*, since this pathogen can be colonized in the oral cavities of mice [33]. However, neither heat-killed bacteria nor LPS can be established in the mouth by single administration. Therefore, we carried out our experiments by the i.v. route.

In this study, we found that i.v. challenge of atherosclerosis-prone hyperlipidemic Apoe[shl] mice with live *Aa*, H.K. *Aa*, or *Aa* LPS accelerated atherosclerosis and increased the plaque lipid content. However, the order of extent of atherosclerosis was live *Aa* > H.K. *Aa* > *Aa* LPS > PBS. The extent of atherosclerotic lesions in *Aa*-challenged mice was significantly greater than that in *Aa* LPS-challenged mice. Furthermore, live *Aa*-challenged mice showed significantly increased aortic expression of TLR2, TLR4, TLR9, and NOD1 compared with PBS-injected controls; the H.K. *Aa* and *Aa* LPS treatment groups exhibited significant increases in TLR4, NOD1, and NOD2 expression.

In a complex tissue such as the atherosclerotic lesion, innate signals can originate from several sources and promote atherogenesis through association with pattern-recognition receptors (PRRs). These signals include various extracellular activation cascades and intracellular signaling pathways and lead to effective clearance of infectious agents and induction of inflammatory responses [13]. TLR4, for example, is the receptor for Gram-negative LPS, as well as other bacterial toxins. TLR2 interacts with the largest variety of pathogenic structures, including bacterial lipoproteins, peptidoglycan, and lipoteichoic acid. TLR9 recognizes unmethylated CpG motifs in bacterial DNA. Therefore, live *Aa* could induce the expression of TLR2, TLR4, and TLR9, due to their surface expression of several pathogen-associated molecular patterns. In contrast, LPS significantly increased TLR4, but not TLR2 and TLR9 expression. Furthermore, previous studies have implicated NLR inflammasomes in the responses to a wide range of microbial pathogens, inflammatory diseases, cancer, and metabolic and autoimmune disorders [34]. The immediate responses of NODs to LPS could also result from NF-κB

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activation *via* TLR signaling [35]. Since it activates multiple PRRs, live *Aa* may induce a more significant inflammatory response than H.K. *Aa* and *Aa* LPS.

Lipid peroxidation (LPO) plays an important role in many diseases. In particular, oxidation of LDL may be a key step in the development of atherosclerosis [12]. Ox-LDL is incorporated into macrophages *via* receptor-mediated endocytosis, leading to the transformation of macrophages into foam cells and the formation of atherosclerotic plaques. 4HNE is a lipid peroxidation product of polyunsaturated fatty acids formed during the oxidation of LDL or membranes [36]. Oxidized phosphatidylcholine (oxPC), the most abundant oxidized phospholipid in ox-LDL, is a specific substrate for PLA2 [37]. NADPH oxidase and MPO are the most important superoxide (O_2-)-producing enzymes in hypertension and atherosclerosis [38, 39]. RAGE and Cav-1 have been implicated in the activation of oxidant stress and inflammatory pathways [40, 41]. Therefore, the increase in ox-LDL level and accompanying increase in NADPH expression or oxidative stress in the aorta of *Aa*-challenged mice suggested that *Aa* plays a important role in oxidative stress and oxidization of LDL. Indeed, periodontitis patients have increased levels of LPO in plasma, saliva, and gingival crevicular fluid [42], and these levels have been cor-related with the severity of periodontal disease [42, 43].

Since atherosclerosis is a chronic inflammatory disease, implications from the results of a three-week experiment in mice may be difficult to determine. However, the Apoe[shl] mice were hyperlipidemic and Apo-E-deficient; therefore, the inflammatory markers had already increased [44], and the symptoms of arteriosclerosis were shown by the progress of time regardless of infection. Therefore, we investigated the involvement of *Aa* or *Aa* components in the early development of arteriosclerosis using the Apoe[shl] mice in a chronic inflammatory state.

The Th1/Th17/Treg balance controls inflammation and may be important in atherosclerosis. To investigate whether the Th1/Th17/Treg functional imbalance existed during *Aa*-accelerated atherogenesis in mice, we detected Th1/Th17/Treg distribution comparatively between *Aa*-challenged atherosclerosis-prone

hyperlipidemic Apoe[shl] mice and sham-treated mice.   In this study, we demonstratd that the percentages of Th17 and Th1 cells was time-dependently increased in the spleen of *Aa*-challenged mice compared with that of PBS-treated mice. In contrast, the ratio of Treg cells in *Aa*-challenged mice was decreased in 14 weeks after the transient increase in 13 weeks although the value was higher than PBS-treated control.

Aa-challenge significantly increased in splenic IL-17$^+$CD4$^+$ T cell population, Th-17- related serum cytokines (IL-17 and IL-6), and Th17-related molecule expressions (IL-6, IL-17RA, IL-21 and IL-23) in spleen. *Aa*-challenge also increased IFN-γ$^+$ CD4$^+$ T cell population in FACS analysis associated with increase in serum IFN-γ and Th1-related molecule expression (STAT4 and IFN-γ) except for T-bet, although the difference was not statistically significant. Although splenic Treg cell ratio immediately declined after transient increase at 13 weeks, the Treg ratio higher than control was maintained even after bacteria-challenged. Indeed, serum IL-10 level and splenic Foxp3 expression was also higher in *Aa*- challenged mice than control mice. These results suggest that the increase in Th1 and Th17 cells rather than the population of Treg cells, is related to exacerbation of arteriosclerosis in *Aa*-challenged mice. Recently, important role of Th1 and Th17 cells in atherosclerosis has reported [45]. Circulating IL-17 and IFN-γ were increased in a subset of patients with coronary atherosclerosis [46]. The majority of pathogenic T cells in atherosclerosis was Th1 phenotype to date, producing pro-inflammatory mediators, such as IFN-γ and activating macrophages [47, 48]. Th1-type immune responses are injurious to the atherosclerotic process. IFN-γ inhibits the synthesis of collagen by the vascular smooth muscle cells, incubating the unstable plaque with thin fibrous cap. It also activates monocytes/macrophages and dendritic cells, maintaining the pathogenic Th1 response [49]. Deficiency in IFN-γ or in its receptor significantly reduces lesion development and enhances plaque collagen content, whereas exogenous administration of IFN-γ enhances lesion development [50, 51]. Recently, IL-17 producing Th17 cells are shown to be an important effector cells in autoimmune and

inflammatory disease in mice [52]. Although the precise role of IL-17 in atherosclerosis remains controversial, recent studies have begun to provide more direct evidence that IL-17 seems to be predominantly proatherogenic [53]. A proof of IL-17 proatherogenic effect is the observation that $CD4^+$ T cells isolated from atherosclerotic coronary vessels express both IL-17 and IFN-γ [54]. Furthermore, increased frequencies of circulating Th17 cells and Th17-associated cytokines correlated to the severity and progression of carotid artery plaques [55].

In addition to Th17 cells, Treg cells are also involved in the regulation of the immuno-inflammatory responses in atherosclerosis. They have anti-inflammatory, immunoregulatory, and suppressive properties and are involved in the modulation of adaptive immune responses, being able to suppress effector $CD4^+$ and $CD8^+$ T cells and to induce tolerance [56, 57]. In this experiment, since the Treg cell was maintaining the ratio higher than control, it is considered that Treg cells do not to participate in exacerbation or suppression of arteriosclerosis directly. In contrast, it has been reported that the conversion of $CD4^+CD25^+$ regulatory T-cells (Tregs) into T-cells with a Th17 phenotype occurs by appropriate inflammatory stimuli [58, 59]. Furthermore, the presence of $IL-17^+/Foxp3^+$ double-positive cells was also recognized in periodontitis [60]. Therefore, the increase in the Foxp3 expression in spleen cell of *Aa*-challenged mice and the increase in $CD4^+Foxp3^+$ T cells for 13 weeks may be the Treg cells before conversion to Th17 cells.

Recently, it was shown that innate immune signal is deeply concerned with Th17 cell differentiation [61]. NOD1 and NOD2 are required to induce early Th17 responses to bacterial pathogens [62]. Since *Aa*-challenge significantly increased in NOD1 and NOD2 mRNA expression in Apoe^shl mice [28], the recognition of innate and inflammatory signals by dendritic cells via pattern recognition receptor, may activate the intracellular pathways involved in $CD4^+$ T cell differentiation into Th17 cells.

7. Conclusion

Our results demonstrate that systemic challenge of Apoe[shl] mice with live *Aa*, H.K. *Aa*, or *Aa* LPS can accelerate atherogenic plaque formation to a greater or lesser degree. Furthermore, the exacerbation of atherosclerosis by *Aa* may involve the enhancement of innate immune signaling and LDL oxidation.

In the next study, our results demonstrated that Th17/Th1/Treg imbalance existed in *Aa*-challenged mice may be contributed to the aggravation of atherosclerosis caused by this parasite. Furthermore, the increase in Th17 by *Aa*-challenge may play a central role in both the induction and persistence of chronic inflammation in part by producing proinflammatory cytokine IL-17.

Acknowledgments

8. References

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Table 1

Primers sequences used for the real-time RT-PCR

Primer	Forward	Reverse
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
TLR2	GGACGTTTGCTATGATGCCTTTG	ACGAAGTCCCGCTTGTGGAG
TLR4	GGGCCTAAACCCAGTCTGTTTG	GCCCGGTAAGGTCCATGCTA
TLR9	ACCAATGGCACCCTGCCTAA	CGTCTTGAGAATGTTGTGGCTGA
NOD-1	AGAGTGCTTCGCTGAGATGCTG	GGCTGTGATGCGATTCTGGA
NOD-2	AGCTTGATGGAACGTGAGGTGTC	GCCAGAGGCCAGCAACATAGTAA
NOX-1	AAGCCATTGGATCACAACCTCAC	ATCCATGGCCTGTTGGCTTC
NOX-2	GCACTCAAGGCTGGTTCTGGTAA	GCAACACGAAGGTCTGTCTGGA
NOX-4	ATTTGGATAGGCTCCAGGCAAAC	CACATGGGTATAAGCTTTGTGAGCA
Cav1	AGGCCAGCGTGTCTATTCAGTTTC	TGAGCTCCTAAATCATCCCAGTCAG
RAGE	AATTGTGGATCCTGCCTCTG	CAGCTCTGACCGCAGTGTAA
iNOS	CGAAACGCTTCACTTCCAA	TGAGCCTATATTGCTGTGGCT
IL-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTGTTCATAC
TGF-β	GTGTGGAGCAACATGTGGAACTCTA	TTGGTTCAGCCACTGCCGTA
IL-17RA	CCGACCTCTTCAACATCACCTC	CCACTCCTGGAACCTAAGCACA
IL-21	AATTCAATGCAGCACAGGCTAAGA	GTTCCCACCCACAGTGAACAATAA
IL-23	ACATGCACCAGCGGGACATA	CTTTGAAGATGTCAGAGTCAAGCAG
STAT3	CCTGGGTTAACCACCCATAGTGAG	AGTGCAGTGGCCAGAACACAAG
T-bet	TTCCCATTCCTGTCCTTCAC	CCTCTGGCTCTGCATCATTC
STAT4	GCATGGGCATCCATCATTTG	AATTGCCAGCTCATCACTTCCAG
IFN-γ	CGGCACAGTCATTGAAAGCCTA	GTTGCTGATGGCCTGATTGTC
FoxP3	AGTGCCTGTGTCCTCAATGGTC	AGGGCCAGCATAGGTGCAAG
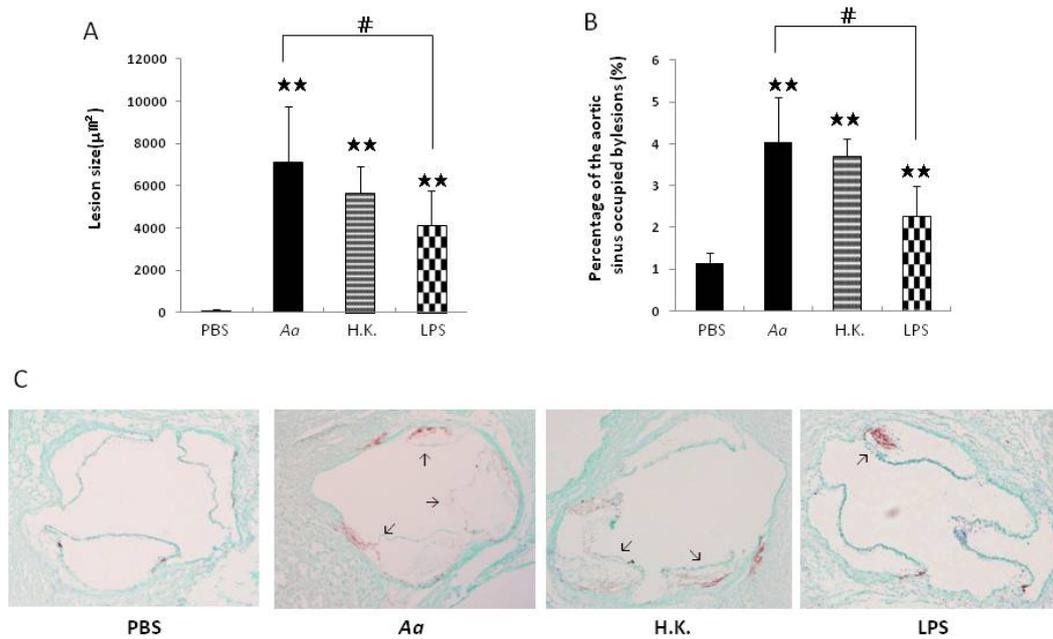
Figure 1. Atherosclerotic plaque formation in the aortic sinuses of Apoe[shl] mice challenged i.v. with live *Aa*, H.K. *Aa*, or *Aa* LPS. Results of histomorphometric analysis of mean lesion area (A) and percentage of the aortic sinus occupied by lesions (B) at 15 weeks. Values represent the mean ± SD ($n = 6$). ★★$p < 0.01$ compared to control mice. #$p < 0.05$ compared with *Aa*-challenged mice. (C) Oil Red O-stained proximal aorta cryosections. Arrow: typical lipid-rich atherosclerotic area stained with Oil Red O. H.K., heat-killed *Aa*.
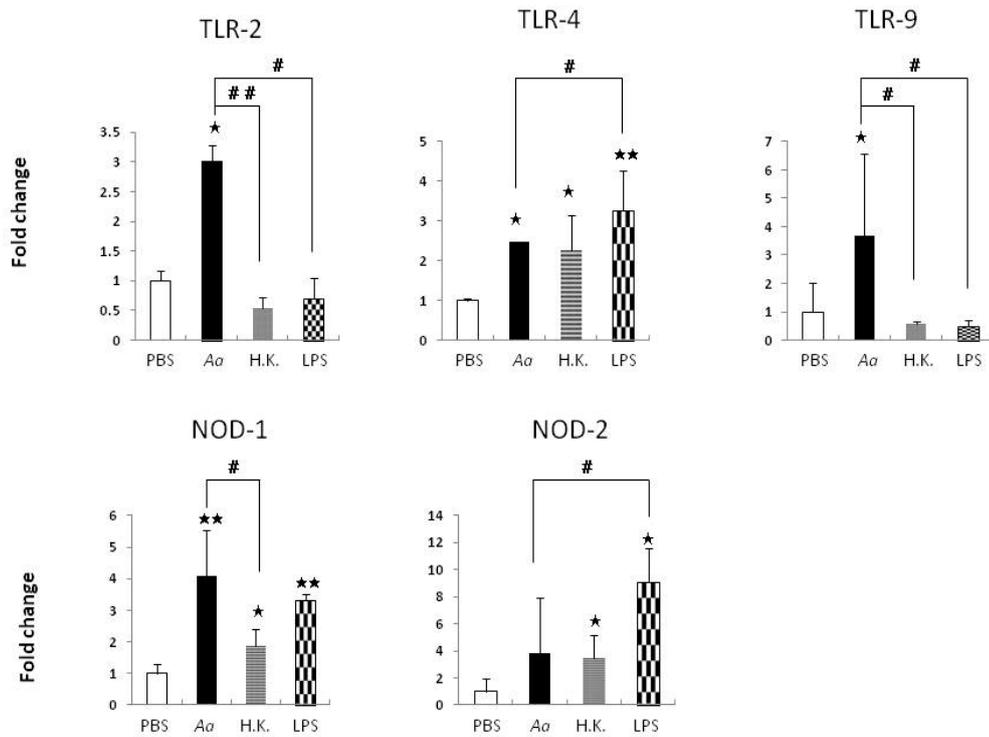
Figure 2. Aortic expression of TLR and NLR in Apoe^{shl} mice 15 weeks following challenge with live *Aa*, H.K. *Aa*, or *Aa* LPS. Relative mRNA levels (normalized to GAPDH) were determined by real-time RT-PCR. Data are expressed as fold-increase in mRNA level compared with sham-inoculated negative controls. Values represent means ± SD ($n = 6$). ★★$p < 0.01$, ★$p < 0.05$ compared with the PBS-treated group. ## $p < 0.01$, # $p < 0.05$ compared with the *Aa*-challenged group. H.K., heat-killed *Aa*.
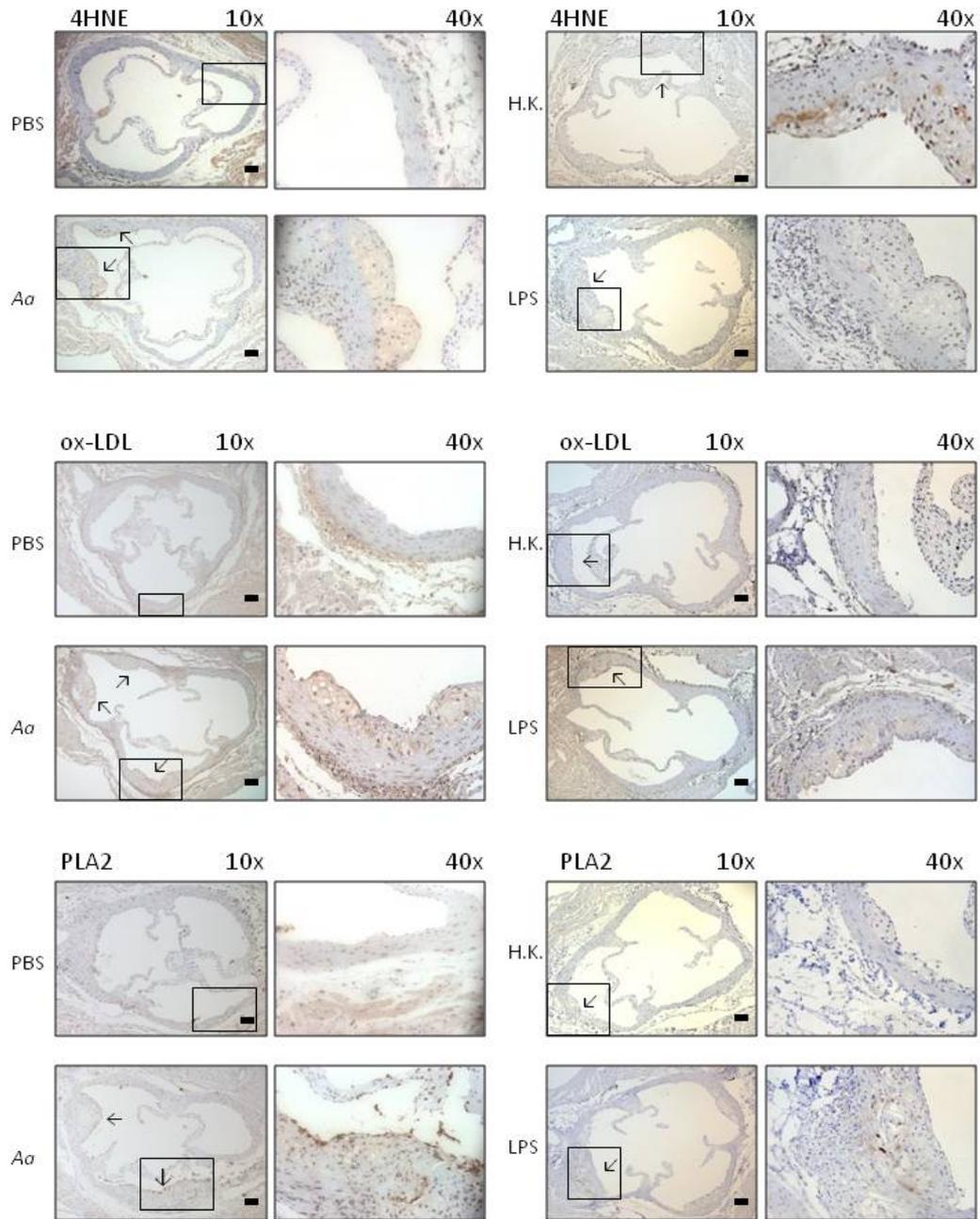
Figure 3. Immunohistochemical analysis of 4HNE, ox-LDL, and PLA$_2$ in the aortic sinus of Apoe^{shl} mice challenged i.v. with live *Aa*, H.K. *Aa*, or *Aa* LPS. Arrow: marker-positive-stained area. Scale bars = 50 μm.
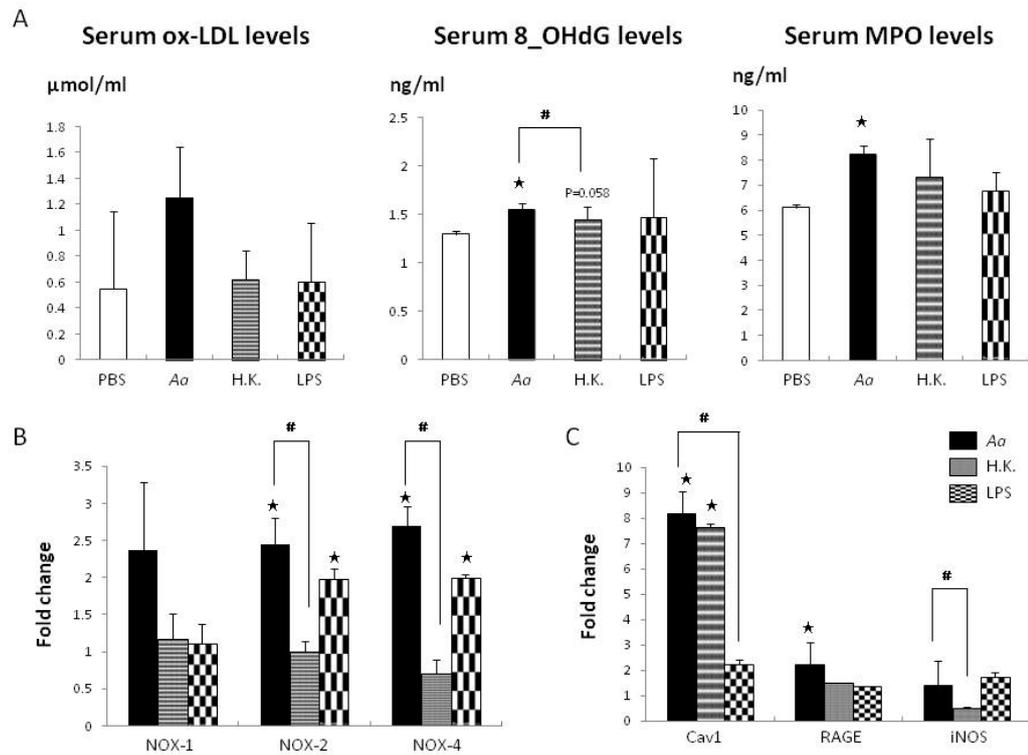
Figure 4. Serum ox-LDL, 8-OHdG, and MPO levels in *Aa*-challenged mice 15 weeks following challenge with live *Aa*, H.K. *Aa*, or *Aa* LPS. (**A**) Aortic expression of NADPH oxidase (**B**) and Cav-1, RAGE, and iNOS (**C**) in Apoe^{shl} mice at 15 weeks. Relative mRNA levels (normalized to GAPDH) were determined by real-time RT-PCR. Values represent means ± SD (n = 6). ★$p < 0.05$ compared with the PBS-treated group. #$p < 0.05$ compared with the *Aa*-challenged group.
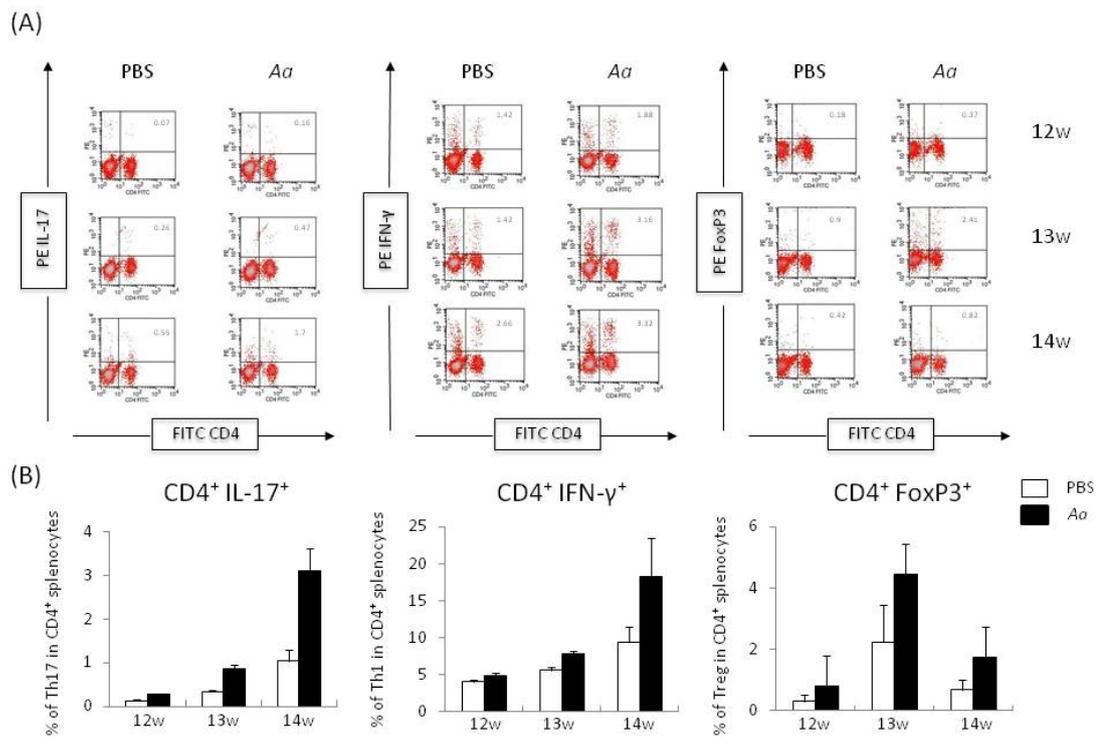
Figure 5. The percentage of Th17, Th1 and Treg cells in total splenocytes (A) and CD4$^+$ splenocytes (B) from Apoe^{shl} mice after *Aa* challenge. At 12, 13 and 14 weeks, spleens were removed, single cell suspensions were prepared, and the cells stained with FITC-conjugated CD4, PE-conjugated IL-17, PE-conjugated IFN-γ, and PE-conjugated Foxp3 Abs were analyzed by flow cytometry. (A) The percentage of positive cells was shown in each panel. (B) Data were presented as the means±SD (n=6).
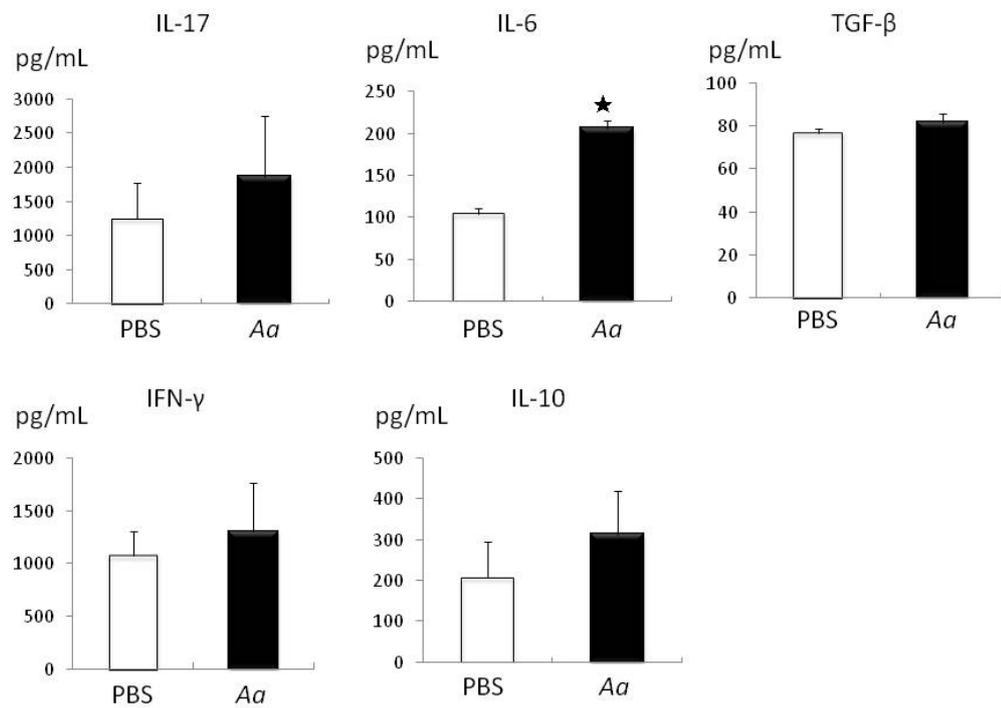
Figure 6. Serum IL-17, IL-6, TGF-β, IFN-γ and IL-10 levels in 15-week Apoe[shl] mice following challenge with *Aa* Values represent means±SD (n=6). ★$p < 0.05$ compared with control mice.
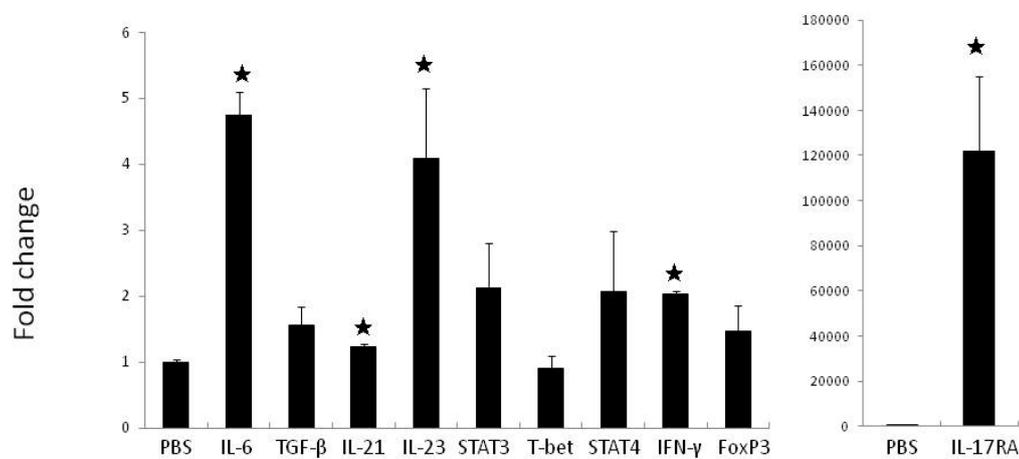
Figure 7. Th17-, Th1- and Treg-related gene expression in splenocytes from 15-week Apoe[shl] mice following challenge with *Aa* Relative mRNA levels were obtained after normalization to GAPDH ($n = 6$) by real-time RT-PCR. The data are expressed as fold increases in mRNA level compared with the PBS-treated negative control. Values represent means ± SD (n=6) . ★$p < 0.05$ compared with control mice.