Atheroprotective Role of Interleukin-6 in Diet- and/or Pathogen-associated Atherosclerosis using an ApoE Heterozygote Murine Model

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Abstract

Background—Pathogens have been implicated in the pathogenesis of inflammatory atherosclerosis. Given the pleiotropic role of Interleukin-6 in the regulation of cytokines, lipid homeostasis, vascular remodeling, and apoptosis we hypothesized that IL-6 plays an important role in development and progression to inflammatory atherosclerosis.

Methods and Results—To explore the role of IL-6 in inflammation- and infection-associated atherosclerosis, 10 week-old $ApoE^{+/-}$-IL-$6^{+/-}$ and $ApoE^{+/-}$-IL-$6^{-/-}$ mice fed either high fat diet or regular chow diet were inoculated intravenously, once per week for 14 or 24 consecutive weeks with 50µl live *Porphyromonas gingivalis* (*P.g.*) (10^7CFU) or vehicle (normal saline). Animals were euthanized at 24 weeks of age (14 weeks injection) or 34 weeks of age (24 weeks injection). Histomorphometric analysis of atheromatous lesions, en face analysis over the aortic tree, immunohistochemistry for macrophages and smooth muscle cell, TUNEL staining for apoptotic cells, serum amyloid A (SAA) levels, serum lipids and glucose level, serum cytokines were obtained. $ApoE^{+/-}$-IL-$6^{-/-}$ mice showed a significant increase in atheromatous lesions in proximal aorta and aortic tree compared to $ApoE^{+/-}$-IL-$6^{+/-}$ mice for all conditions (chow diet and *P.g*-inoculated, high fat diet and *P.g*-inoculated, high fat diet and vehicle-inoculated) at 14 weeks and greater at 24 weeks. SAA levels from $ApoE^{+/-}$-IL-$6^{-/-}$ mice were significantly higher than $ApoE^{+/-}$-IL-$6^{+/-}$ mice. IL-6 deficiency led to profound changes in plaque composition evidenced by increased macrophage infiltration, apoptosis, lipid content and decreased smooth muscle cell mass reflecting an unstable plaque phenotype. Array analysis revealed increased levels of proinflammatory cytokines in $ApoE^{+/-}$-IL-$6^{-/-}$ mice compared to $ApoE^{+/-}$-IL-$6^{+/-}$ mice, irrespective of diet or inoculation.

Conclusion—The genetic deficiency of IL-6 was found to enhance the formation of diet- and/or pathogen associated atherosclerotic plaques and suggests that IL-6 may play an atheroprotective role.

Keywords

Atherosclerosis; Cytokine; Infection; Inflammation; Porphyromonas gingivalis

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INTRODUCTION

Atherosclerosis, which causes ischemic cardiopathy and stroke, is the most common cause of mortality and morbidity in developed countries. High levels of LDL-cholesterol or oxidized-LDL, free radicals (often caused by smoking), hyperglycemia, as well as infectious agents (e.g. *Chlamydia pneumonia, Porphyromonas gingivalis* (*P.g.*), herpes viruses) have been suggested to be implicated in the pathogenesis of atherosclerosis [1–3]. Atherosclerosis results from multiple interactions between injurious stimuli and the healing responses of the arterial wall. The starting point is functional endothelial damage, secondary to mechanical or vascular insult, which is followed by an inflammatory cascade that involves humoral (cytokines, growth factors) and cellular (increased chemotaxis, adherence and infiltration of inflammatory cells) mechanisms. These factors combine in an unbalanced and progressive manner that leads to the final fibroproliferative response [4]. After endothelial injury, direct cell-cell interaction and secretion of chemotactic and growth factors occur, resulting in recruitment of monocytes to subintimal regions, smooth muscle cell proliferation, and increased synthesis of matrix proteins. The recruited monocytes become macrophages, accumulate lipid, and ultimately become foam cells [5, 6]. Although all of the potential triggers of inflammation are not fully known, cytokines, oxidized lipoproteins, and local (arterial) and distant infections such as chronic periodontitis and pneumonia have been implicated in atherogenesis. Vascular cells, including smooth muscle, endothelium, and leukocytes which constitute various atherosclerotic lesions, can produce as well as respond to cytokines such as IL-6, TNF-α, IL-1α, and MCP-1[7]. Among them, IL-6 (a 26 kDa cytokine), produced by lymphocytes, monocytes, fibroblasts, vascular smooth muscle cells, and endothelial cells has been identified as a local and circulating marker of coronary plaque inflammation[8]. It is expressed by lipid-laden macrophages, known as foam cells, and smooth muscle cells in fatty streaks. It is also expressed in the cap and shoulder regions of atheromatous plaques, suggesting an important role in atherogenesis[9]. Furthermore, IL-6 is an important stimulator of the acute phase reaction, acting on both the liver and hypothalamus [10, 11] leading to release of X-reactive protein, serum amyloid A (SAA), and fibrinogen, all known to have proinflammatory roles [12]. However, IL-6 is a pleiotropic cytokine and its role in the modulation of inflammation-related processes, particularly cytokine responses and tissue inflammatory cell infiltration, remains equivocal. Indeed, recently, IL-6 deficiency was reported to result in an enhanced formation of atherosclerotic lesions, reduced collagen metabolism, and elevated levels of serum cholesterol in an ApoE$^{-/-}$ model of atherosclerosis [13]. However weekly, super-physiological injections of recombinant IL-6 promoted a ~2-fold increase in lesion development in ApoE$^{-/-}$ mice, known to rapidly develop extensive atherosclerotic lesions throughout their vascular tree [14]. To our knowledge, the role of IL-6 in bacteria enhanced atherogenic process has never been addressed and warrants further investigation Recent studies in animal models from our group and others have shown that chronic oral infections with microorganisms such as *P.g.*, a pathogen involved in chronic periodontal diseases, increase the risk for coronary vascular disease[15–17]. The present study was conducted to establish the role of IL-6 in diet- and/or bacteria-enhanced atherosclerosis using a IL-6- deficient mice rendered susceptible to atherosclerosis (ApoE$^{+/-}$) where inflammation is a major component.

METHODS

Mice and Diets

The Institutional Animal Care and Use Committee of Boston University approved all animal protocols. In this study, three strains of mice were obtained from Jackson Laboratories (Bar Harbor, ME) as breeding mice. These were apolipoprotein E deficient homozygote (ApoE$^{-/-}$), wild type C57BL/6J hybrids, and IL-6 knock-out (IL-6$^{-/-}$) mice. Female ApoE$^{-/-}$ and male wild-type C57BL/6J hybrids were crossed to generate apolipoprotein E

deficient heterozygotes (ApoE$^{+/-}$). ApoE$^{-/-}$-IL-6$^{-/-}$ mice were generated by crossing male ApoE$^{-/-}$ with female IL-6$^{-/-}$ mice. ApoE$^{-/-}$- IL-6$^{-/-}$ were bred with wild-type C57BL/6J to generate ApoE$^{+/-}$-IL-6$^{+/-}$ mice. After an 8-week growing period, male ApoE$^{-/-}$-IL-6$^{-/-}$ mice were backcrossed with female IL-6$^{-/-}$ mice to generate ApoE$^{+/-}$-IL-6$^{-/-}$. The nature of all the strains was verified by polymerase chain reaction method (genotyping protocols from Jackson Laboratory). The experimental protocols are shown in Fig. 1. Two strains of experimental mice, including ApoE$^{+/-}$-IL-6$^{+/-}$ and ApoE$^{+/-}$-IL-6$^{-/-}$ were weaned at 4 weeks of age, and randomly assigned to either a high fat diet (test diet) containing 1.25% cholesterol, 15% fat (No. 21539, Test Diet) or regular mouse chow (lab diet) containing 0.02% cholesterol and 4.5% fat (rodent diet 5001).

Bacterial strain, dose and route of inoculation

P. g A7436 human isolate was grown on anaerobic agar plates as described previously [18]. We used live bacterial inoculation (10^7 CFU) because in preliminary experiments heat killed *P. g.* failed to produce atherosclerosis. The dose (10^7 CFU) and delivery route (intravenous) of the pathogen compares well with bacteremia encountered in human after dental infection, periodontal surgery, scaling, tooth extraction and flossing. [19–22]

Animal Grouping and Experimental Time Schedule

Mice heterozygous for Apolipoprotein E (ApoE$^{+/-}$) with susceptibility to atherosclerosis were generated as they do not develop spontaneous atherosclerosis [15, 18]. Four week old ApoE$^{+/-}$-IL-6$^{+/-}$ and ApoE $^{+/-}$-IL-6$^{-/-}$ mice fed either HFD or regular chow diet for 6 weeks (n=8) and divided into 4 groups (Group 1 was fed a standard chow diet and inoculated with 50µl vehicle saline (CS)); Group 2 was fed a standard chow diet and inoculated with 50µl (10^7CFU) *P.g.*(CP); Group 3 was fed high fat diet and inoculated with 50µl vehicle saline (HS); Group 4 was fed high fat diet and inoculated with 50µl (10^7CFU) *P.g.* (HP). All groups divided in two sets for testing two time points of 14 weeks (set-1) and 24 weeks (set-2) post inoculation in this study (Fig.1). In summary mice from set-1 received 14 tail vein injections of either vehicle or *P.g.* once weekly and set-2 received 24 tail vein injections of either vehicle or *P.g.* once weekly.

Tissue Harvesting and Preparation

After overnight fasting, mice were heavily sedated with inhaled isoflurane (SOLVAY) and exsanguinated from the femoral arteries. The heart and aorta were perfused, separated close to the heart and processed as described previously [18][23]

Lipids and glucose analysis

Serum samples from 14 weeks and 24 weeks mice from all the groups were evaluated for total cholesterol (TC)(Cayman chemicals), HDL, LDL (Wako Chemicals) and Glucose levels (BioAssay Systems) according to manufacturer's instructions.

Morphometric Analysis

a. En face morphometric analysis of the aortic tree: The extent of atherosclerosis in the aortic tree was determined by en face quantification as described previously [23][24]. The data is presented as the percentage of the aorta occupied by the lesions; b. Histomorphometric and histopathological analysis of atheroma lesions in the proximal aorta: Proximal aortic cross-sections for quantitative and histopathological evaluation of atherosclerotic lesions were prepared as described previously [25]. Briefly stated five sections per animal, each separated by 80 µm, were stained (Sudan IV), counterstained (hematoxylin), and assessed with a computer-assisted image analysis. The total cross-sectional area of the aortic lumen and lesion from the

five images were measured using Image ProPlus 4.0. The value was averaged and then the percentage lesion was expressed as a ratio of total lesion size / total sinus area.

Immunohistochemistry

Briefly, five sections of the proximal aorta per animal, each separated by 80 µm were fixed with ice cold acetone for 10 minutes and incubated for an hour at room temperature with primary antibody to MOMA-2 (1:25) (Chemicon) or alpha smooth muscle actin (1:50) (Σ). Samples were then developed using the ABC elite kit (vector labs) and staining revealed by texas red conjugated avidin (vector labs). Nonimmune IgG from appropriate species were used as negative controls. Stained Macrophages or smooth muscle cells within atherosclerotic plaques in the aortic sinus were quantified using Image ProPlus 4.0. The ratio of the macrophage or smooth muscle stained area / total plaque area was calculated and plotted as described previously [26]. Apoptosis was detected by TUNEL staining of five frozen section per animal, each separated by 80 µm using DeadEnd flourimetric kit (Promega) according to manufacturer's instructions and expressed as a percentage of positive cells relative to the total number of cells in the field of lesion examined [26]. Samples in absence of the TdT enzyme were used as negative controls.

Cytokine Antibody Array

Serum samples from mice euthanized after 24 weeks were analyzed two times with a cytokine antibody array using RayBio Mouse Cytokine Antibody Array III (RayBiotech, Inc., Norcross, GA). These arrays detected 62 mouse cytokines. For each spot, the net optical density level was determined by subtracting the background optical level from the total raw optical density. The level of each cytokine is represented as a percentage of the positive control and presented as expression of cytokines in the ApoE$^{+/-}$-IL-6$^{-/-}$ group compared to the ApoE$^{+/-}$-IL-6$^{+/-}$ group (table-2).

ELISA

Serum samples were used to determine levels of SAA (Biosource International), VCAM-1, L-Selectin, MCP-1, IL-10, mouse MMP-9, and mouse TIMP-1 (R&D Systems), and VEGF (Biosource International) in 24 week serum samples. Cytokine protein levels were assessed using ELISA kits for mouse MMP-9, and mouse TIMP-1(Quantikine; R&D Systems). Heart tissue fragments were proceeded for protein extraction as previously described[27]. 100µg of protein were used for cytokine, MMP-9 and TIMP-1 detection in the myocardium [28]. Assays were done in triplicates and repeated three times with similar results.

Statistical Analysis

All histomorphometric measurements were blinded to the examiner. All quantitative measurements were confirmed by random analysis of one fourth of the specimens by the same examiner (R>0.92) and by another independent examiner (pathologist) to ensure consistency. The intra-examiner and inter-examiner variation was <10%. A level of $p<0.05$ was considered significant. Extent of atherosclerosis was analyzed by Student's paired 2-tailed t-test.

RESULTS

Body weight, glucose level, total serum cholesterol, LDL, and HDL levels

No significant difference in body weight was observed between all groups. Total serum cholesterol, LDL and glucose levels were higher in ApoE$^{+/-}$-IL-6$^{-/-}$ mice when compared to ApoE$^{+/-}$-IL-6$^{+/-}$ mice only in diet- and or bacterial-challenged animal; Furthermore, HDL levels were also decreased in the in ApoE$^{+/-}$-IL-6$^{-/-}$ mice versus ApoE$^{+/-}$-IL-6$^{+/-}$ only in diet-

and or bacterial-challenged animal; no significant difference was observed in the blood chemistry in chow saline groups (table-1)

En face and histomorphometric analysis of atheroma lesions

Quantitative en face analysis revealed a statistically significant increase in lesion size in ApoE$^{+/-}$-IL-6$^{-/-}$ mice compared to ApoE$^{+/-}$-IL-6$^{+/-}$ mice (HP: 3.0±1.0% to 5.8 ±1.1%; HS: 1.6±0.7% to 2.9±1.0%; CP: 0.59±0.3% to 1.38±0.3%) respectively at 24 weeks (p<0.05) (Fig. 2). A similar pattern was observed at 14 week (data not shown). The histomorphological analysis of Sudan red-stained lesions was used to evaluate the percentage of the aortic lumen occupied by the lesion. We did not observe any lesions in the chow diet with vehicle mice (CS) irrespective of the IL-6 genotype. However, we observed an increase in the percentage of aortic sinus occupied by the lesions in mice fed with chow diet and inoculated with *P.g* (CP) in ApoE$^{+/-}$-IL-6$^{-/-}$ versus ApoE$^{+/-}$-IL-6$^{+/-}$ the percentage rose from 0.74±0.4% to 3.57±1.2% (p<0.05) at 14 weeks (Fig. 3c) and from 1.52±0.34% to 6.3±1.9% (p<0.05) at 24 weeks (Figs 3 a, b & c). There was an increase in the percentage of the aortic sinus occupied by the lesion in mice fed with high fat diet and receiving vehicle(HS) in ApoE$^{+/-}$-IL-6$^{+/-}$ mice: 1.9±0.29% compared to 5.8±1.17% (p<0.05) in ApoE$^{+/-}$-IL-6$^{-/-}$ mice at 14 weeks (Fig 3f) and 5.6±1.1% compared to 9.6±2.3% (p<0.05) at 24 weeks (Figs 3 d, e & f). A progression of lesion development was observed in mice from 14 weeks to 24 weeks, with significant increases in mice fed with high fat diet and inoculated with *P.g.* (HP). As anticipated, there was an increase in percentage lesion from ApoE$^{+/-}$-IL-6$^{+/-}$ to ApoE$^{+/}$-IL-6$^{-/-}$ in mice fed with high fat diet and inoculated with *P.g* (HP) :3.5±1.1% vs 15.34±3.7 % (p<0.05) at 14 weeks (Fig.3i). The largest and the most significant increase observed was from 12.2±4.01% to 33.3±7.1% (p<0.05), seen after 24 weeks (Fig3 g, h & i).

Immunohistochemical Analysis of Proximal Aorta

We did not observe any changes in mice fed with chow diet and injected with saline. In all the other experimental groups, atherosclerotic lesions exhibited a significant increase in macrophage infiltration in ApoE$^{+/-}$-IL-6$^{-/-}$ mice compared to ApoE$^{+/-}$-IL-6$^{+/-}$ mice (HP: 18.9 ±1.5% to 39.9±4.1%; HS: 15.2±125% to 26.18±4.09%; CP: 5.1±1.1% to 14.7±2.1% respectively (p <0.05) (Fig 4 a, b & c). There was a decrease in smooth muscle cell accumulation in ApoE$^{+/-}$-IL-6$^{-/-}$ mice versus ApoE$^{+/-}$-IL-6$^{+/-}$ mice (fig4 d, e & f). The percentage decreased from 30.2±1.4% to 15.4±1.6%, 19.4±3% to 17.9±1.4% and from 10.4±1.7% to 6.3±2% (p<0.05) in HP, HS and CP mice respectively. Furthermore, the marked increase in the inflammatory component of the lesions in ApoE$^{+/-}$-IL-6$^{-/-}$ mice with high fat diet and inoculated with *P.g.* was associated with a substantial increase in the occurrence of cell death within the plaques in ApoE$^{+/-}$-IL-6$^{-/-}$ mice (8.1±1.0%) versus (4.8±1.2%) in ApoE$^{+/-}$-IL-6$^{+/-}$ (p <0.05) (Fig4 g, h & i)

Increase in Serum level of SAA in ApoE$^{+/-}$-IL-6$^{-/-}$ mice as compared to ApoE$^{+/-}$-IL-6$^{+/-}$ mice

There was a significant increase in the SAA level in ApoE$^{+/}$-IL-6$^{-/-}$ mice as compared to ApoE$^{+/-}$-IL-6$^{+/-}$ mice with high fat diet and inoculated with *P.g.* (HP): 38.3±5.35µg/ml compared to 181.2±15.47µg/ml; (p <0.05). We also observed increases in SAA levels in mice fed with high fat diet with vehicle (HS) from 17.6±4.2µg/ml in ApoE$^{+/-}$-IL-6$^{+/-}$ mice to 67 ±9.33µg/ml in the ApoE$^{+/-}$-IL-6$^{-/-}$ animals; (p <0.05) and in mice with chow diet and with *P.g.* (CP), from 16.3±1.4 µg/ml in ApoE$^{+/-}$-IL-6$^{+/-}$ to 42±8.56µg/ml in ApoE$^{+-/}$-IL-6$^{-/-}$ animals; (p <0.05) (Fig. 5).

Serum proinflammatory cytokine level

Twenty-four week serum samples were analyzed for 62 cytokines (Table 2). Cytokine array data were validated independently by ELISA and found congruent. ApoE$^{+/-}$-IL-6$^{-/-}$ mice

displayed profound increases in most proinflammatory cytokines and chemokines, such as IL-1α, IL-1β, TNF- α, MCP-1, MCP-5, VEGF, L-Selectin, Π-Selectin, VCAM-1, TNFR-I, TNFR-II, M-CSF, and CD40 relative to ApoE$^{+/-}$-IL-6$^{+/-}$ mice only in diet-and or bacterial-challenged animals; no significant difference was observed in the cytokine levels in chow saline groups. The most significant increase was seen in mice with high fat diet and inoculated with *P.g.* (HP). However, none of the groups demonstrated any changes in the anti-inflammatory cytokines such as IL-10 and IL-4. To validate the cytokine array data, selected cytokines (VCAM-1, L-Selectin, VEGF, MCP-1 and IL-10) were measured using the ELISA. The level of cytokines detected using ELISA was consistent with cytokine array data and representative data is shown in table 3.

Total MMP-9 and TIMP-1 levels in serum and heart tissues

Total MMP-9 levels increased significantly by 48%, 53% and 43% and TIMP-1 levels decreased significantly by 22%, 24% and 23% in ApoE$^{+/-}$-IL-6$^{-/-}$ mice when compared to ApoE$^{+/-}$-IL-6$^{+/-}$mice in high fat diet inoculated with *P.g* ,high fat diet injected with vehicle and chow diet inoculated with *P.g* respectively; no significant difference was observed in the chow saline groups (fig-6). ELISA for protein levels in heart tissue exhibited similar results (data not shown).

DISCUSSION

The purpose of this investigation was to establish the role of IL-6 in inflammation and to test the hypothesis that blocking IL-6 signaling by genetic deletion alters the atherogenic process that can be enhanced by bacteria (*P. gingivalis*) or high fat diet in an ApoE$^{+/-}$ murine model. Both enface and histomorphometric data revealed a greater percentage of the aorta and aortic lumen being occupied by the atherosclerotic lesions in ApoE$^{+/-}$-IL-6$^{-/-}$ mice. IL-6-deficient mice fed with high fat diet and inoculated with *P.g.* (HP) exhibited greater lesions compared to mice fed with high fat diet and receiving vehicle (HS) and mice fed with chow diet and inoculated with *P.g.* (CP).

Highly proteolytic organisms like *P.g.* possess important virulence factors (gingipains, fimbrillin peptides, capsule polysaccharides, lipopolysaccharides, outer membrane vesicles and proteases) which influence the balance between pro- and anti-inflammatory mediators [29] and aggravate atherosclerosis [15]. Both the bacterial endotoxins and OxLDL [30, 31] are known activators of cytokine cascades. These cytokines are typically released from stimulated mononuclear phagocytes and augment endothelial cell expression of many vascular cell adhesion molecules implicated in atherogenesis. By augmenting the expression of leukocyte adhesion molecules, bacterial infection and OxLDL could influence the crucial aspects of atherogenesis.

Classical risk factors for atherosclerosis are elevated total cholesterol and LDL and decreased HDL levels [32, 33]. In the present study IL-6-deficiency leads to an increase in the serum total cholesterol and LDL and decrease in HDL levels as compared to ApoE$^{+/-}$- IL-6$^{+/-}$ mice only in diet-and or bacteria-challenged groups. No significant difference in serum total cholesterol, LDL and HDL levels were observed in chow saline groups irrespective of the IL-6 status. Furthermore, it is known that the hyperglycemia can lead to modification of macromolecules, such as forming advanced glycation end products (AGE). By engaging surface receptors such as RAGE (receptor for AGE), AGE-modified proteins can augment the production of proinflammatory cytokines and activate other inflammatory pathways in vascular endothelial cells and lead to increased atherosclerosis [34]. Consistent with the current literature, our data demonstrate that high fat diet and/or *P.g.*, stimulated an elevation in serum glucose which in the absence of IL-6 amplified the atherosclerosis phenotype observed while chow saline animals did not exhibit serum glucose elevation irrespective of the IL-6 status. Altogether the

present data strongly suggest that diet- and or bacterial exposure are responsible for the blood chemistry changes observed irrespective of the IL-6 status.

It has been shown that pro-atherosclerotic factors such as OxLDL, reactive oxygen species, high glucose, and inflammatory cytokines activate apoptotic pathways in cellular elements of the lesion [35–37]. Various studies have demonstrated that vulnerable or unstable plaques are rich in inflammatory cells and exhibit a substantial increase in apoptotic cell death leading to the formation of a highly thrombogenic lipid core [38–40]. Histological analysis of the proximal aorta with specific staining for smooth muscle cells, macrophages and tunnel positive apoptotic cells to determine plaque stability, demonstrated that the composition of advanced lesions was profoundly affected by bacteria-challenged IL-6-deficient mice. IL-6-deficient mice fed a high fat diet and/or inoculated with *P.g.* (HP) resulted in increased macrophage accumulation, decreased smooth muscle cell mass and increased apoptosis in lesion areas as compared to IL-6 heterozygote mice. Thus, we conclude that in wild type animals endogenous levels of IL-6 would contribute to the attenuation of the inflammatory process within the atherosclerotic lesions. This result shows also that high fat diet and/or *P.g.* may initiate the apoptotic pathway in various cellular elements of the plaque explaining the enhanced and unstable atherosclerotic lesions in the IL-6 deficient mice.

One of the earliest pathophysiological changes manifested after an inflammatory stimulus, is altered concentrations of certain plasma proteins synthesized by the liver. Although IL-6 serves as a major mediator, regulating the synthesis of most acute phase proteins, other cytokines such as TNF-α and IL-1 also regulate these proteins[41]. Our observation of significant increase in the SAA levels in IL-6 deficient mice may stem from TNF-α and IL-1 stimulation.

The evidence implicating cytokines as inflammatory mediators in atherosclerosis led us to survey cytokines to determine their involvement in atherosclerotic progression in ApoE$^{+/-}$-IL-6$^{-/-}$ and ApoE$^{+/-}$-IL-6$^{+/-}$ mice under various conditions. Increase in the serum levels of IL-1 α, IL-1 β, MCP-1, MCP-5, TNF-α, VEGF, VCAM-1, P-Selectin, and L-Selectin in IL-6 deficient mice fed with high fat diet and/or inoculated with *Pg* cannot be disregarded, as they play a critical role in progression of atherosclerosis [7, 18, 42].

It has been shown that peripheral blood levels of MMP's are elevated in unstable angina [43]. Our observation that IL-6 may be involved in plaque stability, can be further explained by the reduced level of TIMP-1 in ApoE$^{+/-}$-IL-6$^{-/-}$ compared to ApoE$^{+/-}$-IL-6$^{+/-}$ mice as evidenced in serum and heart tissue. Indeed it is known that reduced TIMPs stimulate oxidative stress and promote gelatinolytic MMP activity in vivo thereby favoring unstable plaque[44]. The increased MMP activity observed during atherosclerotic plaque development and instability was shown to stem from an increased cytokine and growth factor-stimulated gene transcription, elevated zymogen activation and an imbalance in the MMP: TIMP ratio [45]. Our data demonstrate an increase in MMP-9 and a decrease in TIMP-1 levels in serum and heart tissue of diet and/or *P.g* inoculated ApoE$^{+/-}$-IL-6$^{-/-}$ mice compared to the ApoE$^{+/-}$-IL-6$^{+/-}$ mice and suggest that IL-6 deficiency may shift the equilibrium of TIMP-1 and MMP-9 toward a more unbalanced MMP activation thereby promoting unstable atherosclerotic plaques.

Of most prominence was that, in stark contrast to proinflammatory cytokines, the levels of IL-10 in ApoE$^{+/-}$-IL-6$^{-/-}$ mice were similar to those in ApoE$^{+/-}$-IL-6$^{+/-}$ mice. This suggests that a balanced interplay between proinflammatory and anti-inflammatory mediators (IL-6 and IL-10) may be involved in the perpetuation of atherosclerosis within the vessel wall, and may modulate the development of atherosclerotic plaques. The mechanism may involve recruiting inflammatory cells to the plaque which implies that one function of IL-6 is to suppress the level of proinflammatory cytokines without compromising the level of anti-inflammatory cytokines.

Therefore the absence of IL-6 results in more pronounced response of proinflammatory cytokines.

Conclusions

Lifetime deficiency of IL-6 in the ApoE$^{+/-}$ murine model results not only in enhanced but also unstable atherosclerotic lesion formation, both in response to a high fat diet and/or weekly challenge with live *P.g.* IL-6 is suggested to have an atheroprotective role in diet-and/or bacteria-challenged animals.

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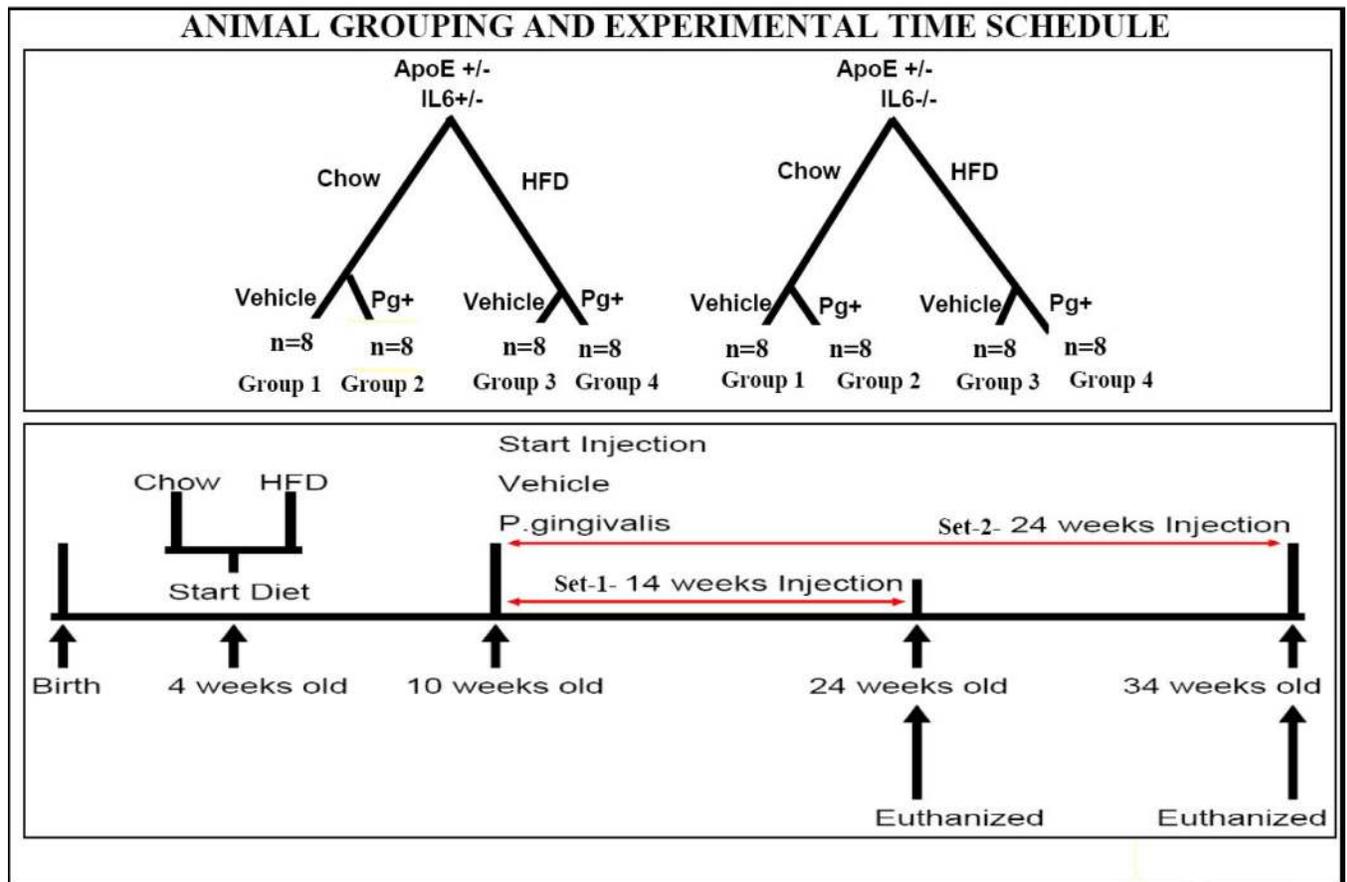
Figure 1.
Four week old ApoE$^{+/}$-IL-6$^{-/-}$ and ApoE$^{+/}$-IL-6$^{+/-}$ mice fed either HFD or regular chow diet for 6 weeks were divided into 4 groups {Group 1 was fed a standard chow diet and inoculated with 50µl vehicle (CS) (normal saline); Group 2 was fed a standard chow diet and inoculated with 50µl (10^{7}CFU) *P.g.*(CP); Group 3 was fed high fat diet and inoculated with 50µl vehicle (HS) (normal saline); Group 4 was fed high fat diet and inoculated with 50µl (10^{7}CFU) *P.g.* (HP)}. All groups were divided in two sets for testing two time points of 14 weeks (set-1) and 24 weeks (set-2) post inoculation in this study. In summary mice from set-1 received 14 tail vein injections of either vehicle or *P.g.* once weekly and set-2 received 24 tail vein injections of either vehicle or P.g. once weekly.
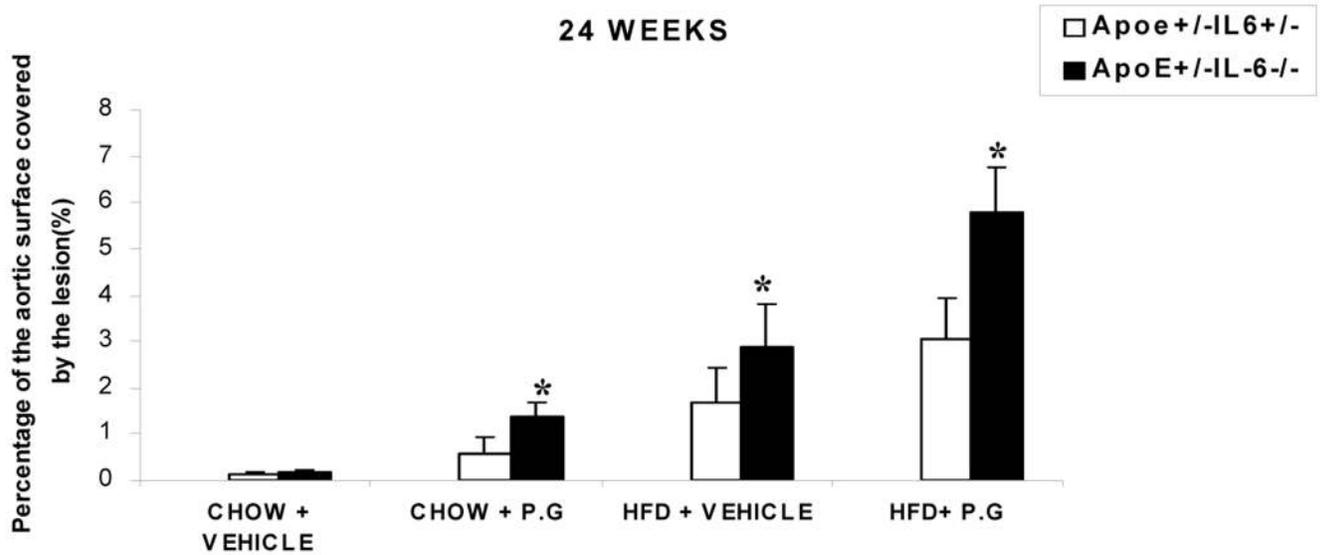
Figure 2.
En face analysis: percentage of aortic surface area covered by lesions in chow-fed or HFD groups for 2 genetic background mouse groups (ApoE$^{+/-}$-IL-6$^{-/-}$ and ApoE$^{+/-}$-IL-6$^{+/-}$) inoculated with *P gingivalis* or vehicle for 24 weeks. Values represent mean±SD, *p <0.05 for ApoE$^{+/-}$-IL-6$^{-/-}$ mice as compared to ApoE$^{+/-}$-IL-6$^{+/-}$ mice in same condition on same diet. Abbreviations are as defined in text.
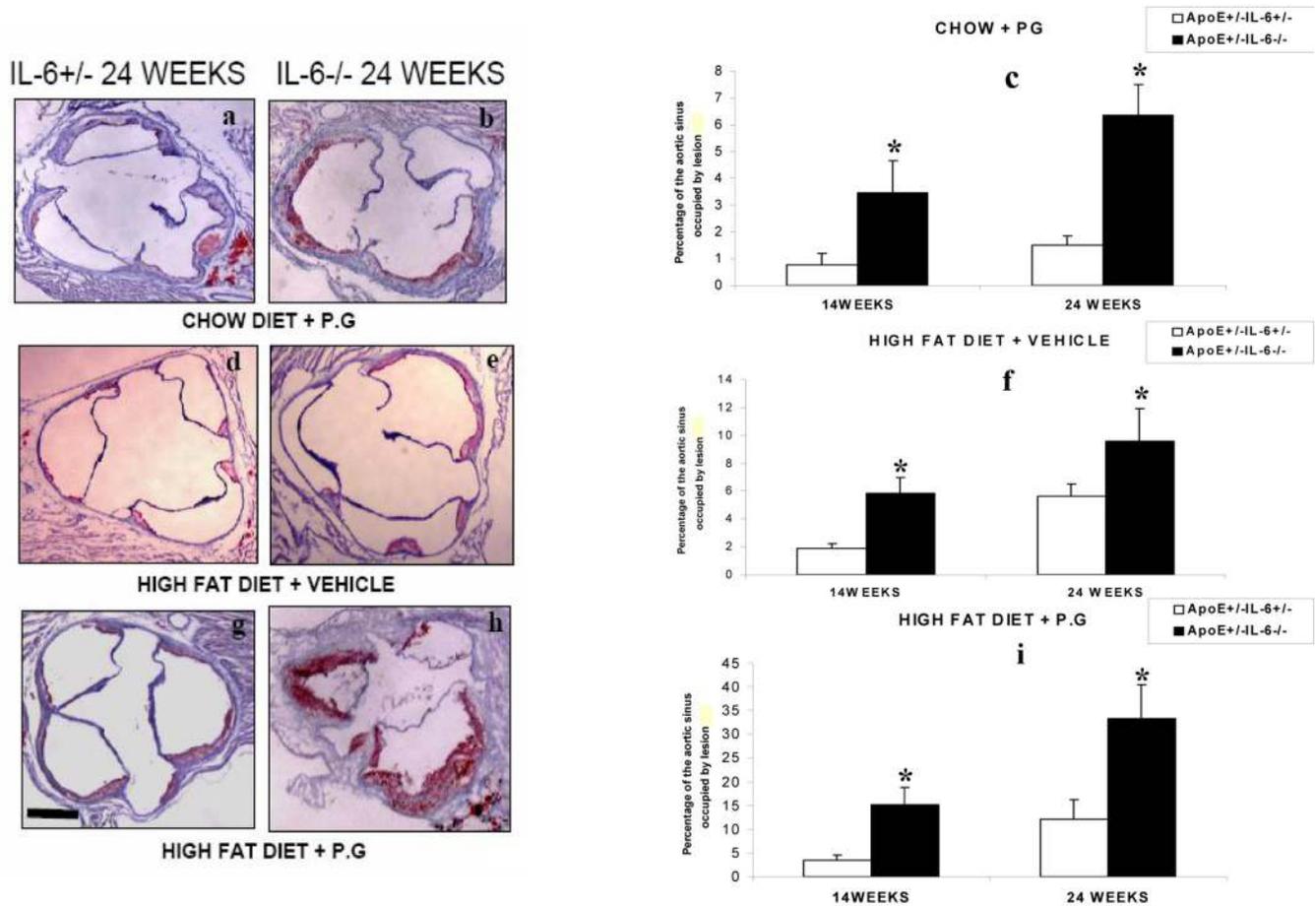
Figure 3.
Microscopic cross sections (10 µm) of the proximal aortic root were stained with sudan and counterstained with hematoxylin to reveal lipid deposition and quantified by digital morphometry for both ApoE$^{+/-}$- IL-6$^{-/-}$ mice and ApoE$^{+/-}$-IL-6$^{+/-}$ mice maintained on standard chow diet and inoculated weekly with *Pg* (CP)(fig-3c), on high fat diet and inoculated weekly with vehicle (HS)((normal saline) fig-3f) and on high fat diet and inoculated weekly with *P.g.*(HP)(fig-3i) .The data is presented as percentage of total lumen of the proximal aorta occupied by lesions at 14 weeks and 24 weeks. Values represent mean±SD, *p <0.05 for ApoE$^{+/-}$-IL-6$^{-/-}$mice as compared to ApoE$^{+/-}$-IL-6$^{+/-}$ mice in same condition on same diet. Abbreviations are as defined in text. Photomicrographs shown are representative images used to obtain the data presented in (Fig-3c, 3f & 3i), of proximal aortic lesions in ApoE$^{+/-}$-IL6$^{+/-}$ and ApoE$^{+/-}$-IL-6$^{-/-}$ mice maintained on standard chow diet and inoculated weekly *Pg* (CP)(fig-3a & 3b), on high fat diet and inoculated weekly with vehicle(normal saline) (HS) (fig-3d & 3e) and on high fat diet and inoculated weekly with *P.g.*(HP) (fig-3g & 3h) for 24 weeks. Original magnifications 20X. Scale bar represents 0.5mm.
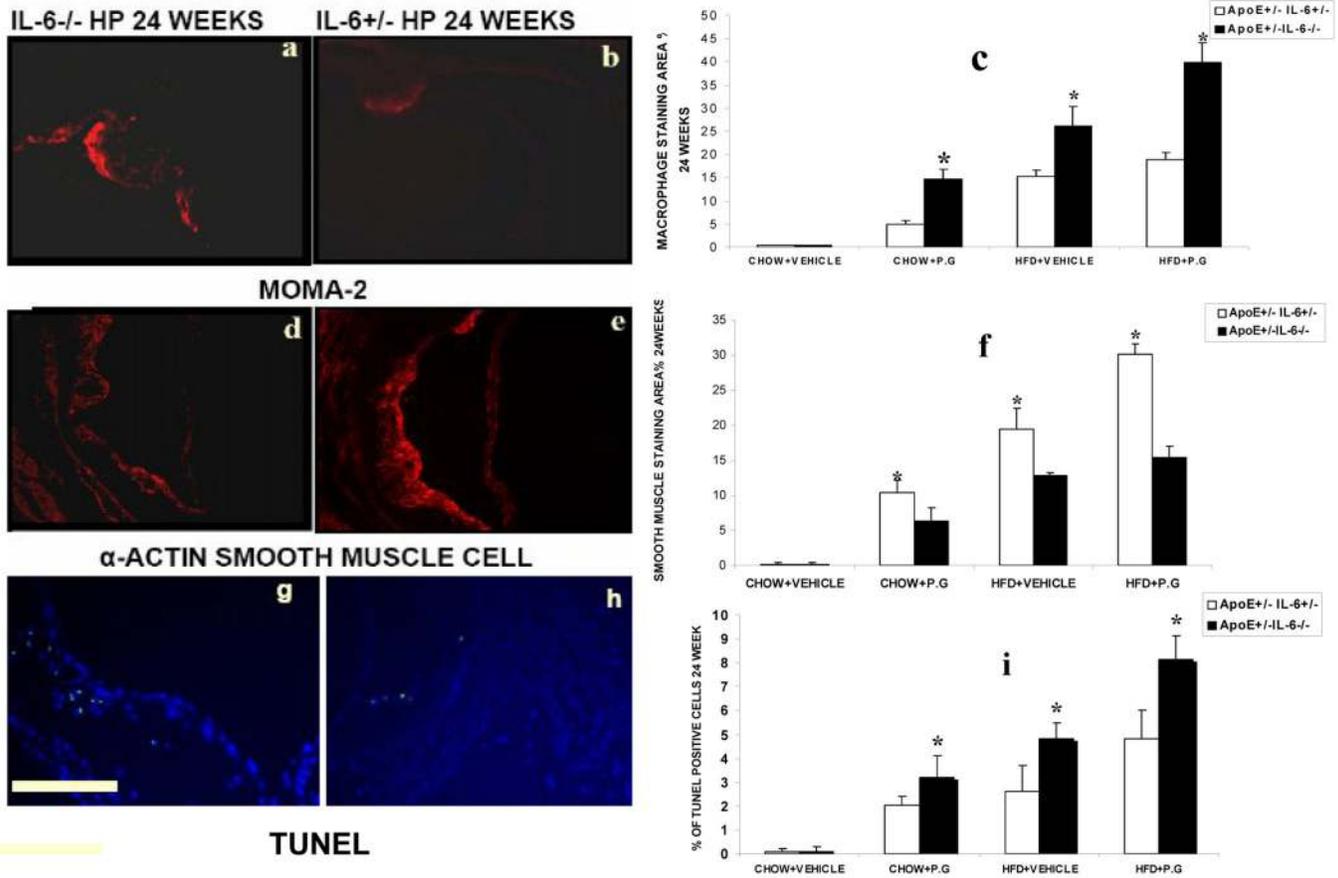
Figure 4.

Representative photomicrographs show immunostained sections of macrophage infiltration (MOMA-2 red staining) (fig-4a & 4b), smooth muscle cell(α-SMA red staining) (fig-4d & 4e) and TUNEL positive cells (green spots coinciding with nuclear stain DAPI)(fig 4g & 4h) in atherosclerotic plaques from the aortic sinus of ApoE$^{+/-}$-IL6$^{+/-}$ and ApoE$^{+/-}$-IL-6$^{-/-}$ mice maintained on high fat diet and inoculated weekly with *P.g* (HP) for 24 weeks. Quantitative computer-assisted image analysis(as described in material and methods) was used to determine the percentage of macrophage-positive areas (fig-4c),smooth muscle cell area(fig-4f) and TUNEL/DAPI positive cells(fig-4i) in proximal aortic lesions in ApoE$^{+/-}$-IL6$^{+/-}$ and ApoE$^{+/-}$-IL-6$^{-/-}$ mice of all the groups at 24 weeks. Data represent mean ± SD. *p<0.05. Original magnifications 100X. Abbreviations are defined in the text. Scale bar represents 0.5mm.

Figure 5.
SAA levels in 24 weeks post inoculation serum samples as determined by ELISA. Data represent mean ± SD. *p<0.05. Abbreviations are as defined in the text.
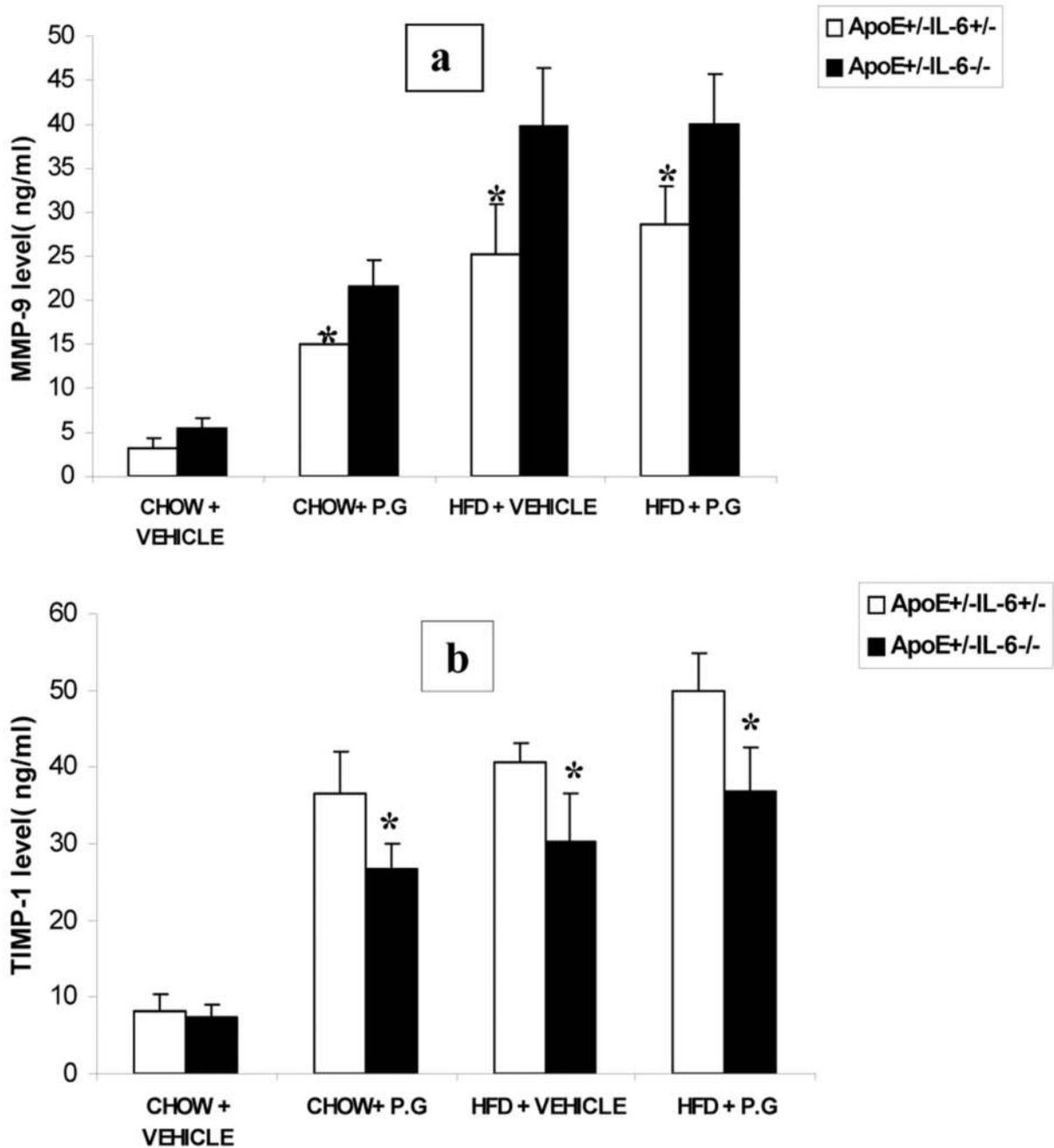
Figure 6.
Level of total MMP-9(6a; ng/ml) and TIMP-1(6b; ng/ml) assessed in serum at 24 week by ELISA. Data represent mean ± SD*p< 0.05.

TABLE-1

Metabolic profile of animals

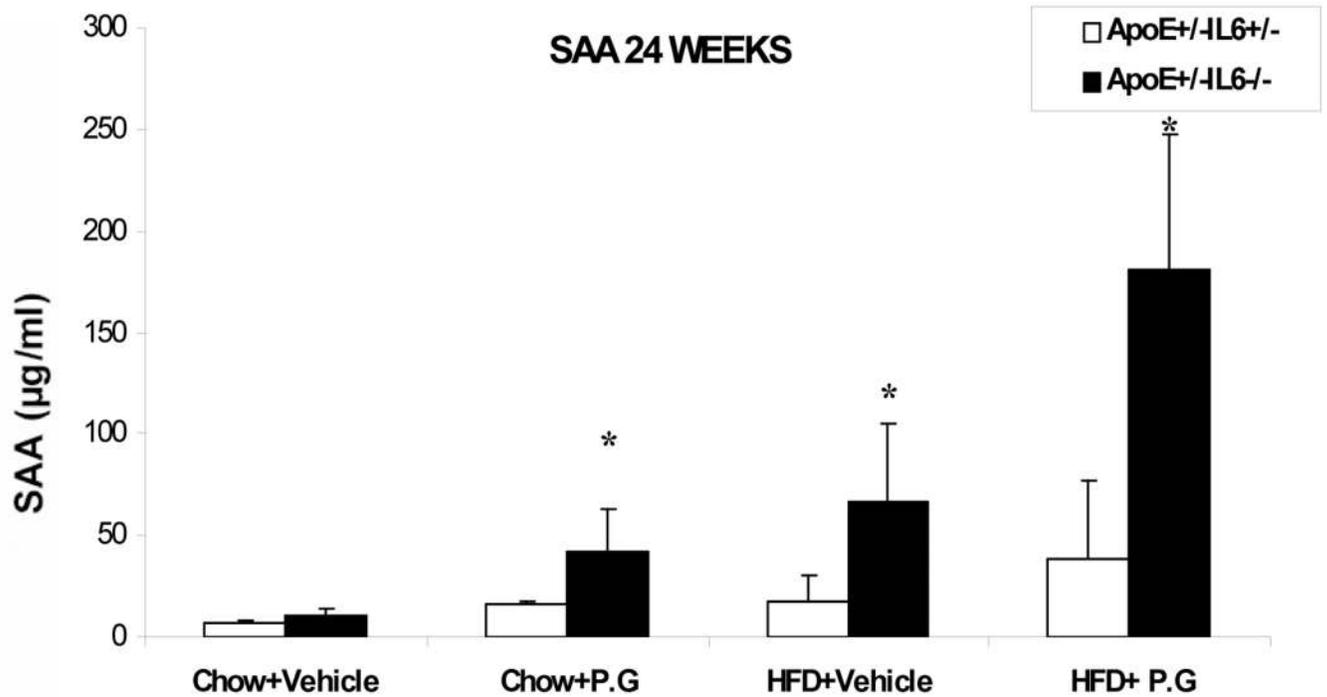|	ApoE$^{+/-}$-IL-6$^{+/-}$	ApoE$^{+/-}$-IL-6$^{+/-}$	P value*	ApoE$^{+/-}$-IL-6$^{+/-}$	ApoE$^{+/-}$-IL-6$^{+/-}$	P values*
1. Serum lipids and glucose level in mice fed with chow diet and receiving vehicle (normal saline)						
Duration (weeks)	14	14		24	24	
Number of mice (n)	8	8		8	8	
Weight (g)	28.1±4	29.3±5		30.3±4	31.3±2	
Total cholesterol (mg/dl)	180.2±12	198.2±11	>0.05	195.8±10	205±15	>0.05
HDL (mg/dl)	34±12	28±5	>0.05	35.3±5	30±8	>0.05
LDL (mg/dl)	41±7	46±4	>0.05	48.5±11	54.1±9	>0.05
Glucose (mg/dl)	78.5±12	87.9±14	>0.05	91.4±16	93.3±3	>0.05
2. Serum lipids and glucose level in mice fed with chow diet and receiving P. gingivalis						
Duration (weeks)	14	14		24	24	
Number of mice (n)	8	8		8	8	
Weight (g)	30.1±4	31.1±2		31.7±3	31.0±3	
Total cholesterol (mg/dl)	201.1±13	258.9±18	<0.05	264.6±10	331.6±11	<0.05
HDL (mg/dl)	67±9	48.9±5	<0.05	56±4	40.6±9	<0.05
LDL (mg/dl)	52±6	69±3	<0.05	59.9±8	84.5±11	<0.05
Glucose (mg/dl)	93.3±5	115.9±12	>0.05	119.3±16	133±10	>0.05
3. Serum lipids and glucose level in mice fed with high fat diet and receiving vehicle (normal saline)						
Duration (weeks)	14	14		24	24	
Number of mice (n)	8	8		8	8	
Weight (g)	30.1±4	29.1±5		30.1±6	31.1±4	
Total cholesterol (mg/dl)	609±11	811.3±10	<0.05	806.13±14	1069.3±13	<0.05
HDL (mg/dl)	106.1±11	88±12	<0.05	99.6±4	70±8	<0.05
LDL (mg/dl)	114.5±6	151±6	<0.05	143.9±5	208.5±9	<0.05
Glucose (mg/dl)	125±7	166.6±9	<0.05	134±10	179±15	<0.05
4. Serum lipids and glucose level in mice fed with high fat diet and receiving P. giugivalis						
Duration (weeks)	14	14		24	24	
Number of mice (n)	8	8		8	8	
Weight (g)	29.1±3	30.1±2		31.1±2	32.1±1.2	
Total cholesterol (mg/dl)	645.67±16	909±11	<0.05	819.13±14	1135±18	<0.05
HDL (mg/dl)	154±6	80.8±11	<0.05	117±10	76±9	<0.05
LPL (mg/dl)	142.8±6	197.2±10	<0.05	168.6±11	249.6±9	<0.05
Glucose (mg/dl)	140.3±13	182.9±14	<0.05	149±21	195±15	<0.05

*
Significance between ApoE$^{+/-}$-IL-6$^{+/-}$ and ApoE$^{+/-}$-IL-6$^{+/-}$ for respective groups. Abbreviattions are as defined in the text.

Table-2

Cytokine profile

Cytokine	Group 2 (CP)	Group 3 (HS)	Group 4 (HP)
IL-1β	++	++	++
IL-2	+	+	+
IL-3	++	+	+
IL-3Rb	+++	++	++
IL-4	−	−	−
IL-5	−	−	−
IL-6	+	+	−
IL-9	+	+	−
IL-10	++	++	+++
IL-12p70	++	++	+
IL-13	−	−	++++
IL-17	+	−	−
KC	−	−	++
LEPTIN-R	++	+	+
LEPTIN	++	++	++
LIX	+	+	+
L-SELECTIN	++	++	++
LYMPHOTACTIN	++	+++	+++
TNFR-II	++	++	++
TPO	+++	++++	++

Cytokine	Group 2 (CP)	Group 3 (HS)	Group 4 (HP)
MCP-1	+++	+++	+++
MCP-5	++++	+++	+++
M-CSF	++	++	++
MIG	+	+	+
MIP-1α	++	++	+++
MIP-1β	++	++	++
MIP-1γ	+	+	+
MIP-2	+++	+	++
MIP-3β	+++	+++	+++
MIP-3α	−	−	−
P-SELECTIN	++	+++	++++
RANTES	++	+++	++
SCE	++	+++	+++
SDF-1α	+++	+++	+++
TARC	++++	+++	++++
TCA-3	++++	++++	+
TECK	+	+	+
TNF-α	+++	+++	++++
TBFR-I	+++	++	+++

...pression of cytokines in the ApoE$^{+/-}$-IL-6$^{-/-}$ group compared to the ApoE$^{+/-}$-IL-6$^{+/-}$ group. Results are presented for subgroups receiving a standard chow diet and inoculated with *Pg* (CP); a high diet and inoculations with vehicle (HS); and a high fat diet and inoculated with *Pg* (HP)

Unchanged

20% INCREASE: +

−40% INCREASE: ++

−60% INCREASE: +++

1% INCREASE: ++++

Table-3

Cytokine level detected using ELISA

	ApoE$^{+/-}$-IL-6$^{+/-}$	ApoE$^{+/-}$-IL-6$^{-/-}$	p-value[*]
Number of mice (n)	8	8	
VEGF (pg/ml)			
CHOW DIET + VEHICLE	30.1±11	41.2±18	>0.05
CHOW DIET + P.G	47.3±33	144.2±49	<0.05
HFD + VEHICLE	58.1±54	223.4±63	<0.05
HFD + P.G	85.0±53	310.6±43	<0.05
VCAM-1 (pg/ml)			
CHOW DIET + VEHICLE	261.4±23	301.3±32	>0.05
CHOW DIET + P.G	352.4±43	965.2±67	<0.05
HFD + VEHICLE	481.3±54	1106±72	<0.05
HFD + P.G	676.6±65	1645±74	<0.05
L-selectin (pg/ml)			
CHOW DIET + VEHICLE	643.2±20	685.3±23	>0.05
CHOW DIET + P.G	718.0±58	2151.5±76	<0.05
HFD + VEHICLE	920.0±76	1909.3±54	<0.05
HFD + P.G	1145.0±34	2558.1±68	<0.05
MCP-1 (pg/ml)			
CHOW DIET + VEHICLE	20.34±11	25.2±10	>0.05
CHOW DIET + P.G	40.4±21	170.5±34	<0.05
HFD + VEHICLE	168.9±45	356.2±65	<0.05
HFD + P.G	242.5±63	549.6±78	<0.05
IL-10 (pg/ml)			
CHOW DIET + VEHICLE	455.2±8.1	464.2±3.4	>0.05
CHOW DIET + P.G	462.4±3.9	468.5±9.8	>0.05
HFD + VEHICLE	469.9±6.5	474.3±7.6	>0.05
HFD + P.G	484.5±8.3	487.3±10.8	>0.05

[*] Significance between ApoE$^{+/-}$-IL-6$^{-/-}$ and ApoE$^{+/-}$-IL-6$^{+/-}$ for respective groups. Abbreviattions are as defined in the text.