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Impact of a CD36 Inhibitor on *Porphyromonas gingivalis* Mediated Atherosclerosis

Running title: AP5055 periodontal disease & atherosclerosis

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Highlights

- CD36 inhibitor, AP5055 abrogates atherosclerotic lesion burden associated with periodontal disease.
- *In vitro* analysis showed that AP5055 inhibited the transcriptional activity of NF-κB.
- AP5055 decreased levels of cholesterol in plasma.
- AP5055 has a role in the reduction of pro-inflammatory cytokines.

Abstract

Objective: To determine if AP5055 drug, an inhibitor of CD36, prevents the increase in *Porphyromonas gingivalis (P. gingivalis)* mediated atherosclerosis in low-density lipoprotein receptor knockout (LDLR KO) mice by targeting CD36.

Methods: Male LDLR KO mice were infected with *P. gingivalis* by oral lavage to induce periodontal disease and fed a western diet to induce atherosclerosis. Mice were treated with the CD36 inhibitor, AP5055 (1mg/kg), or vehicle (1% DMSO). Aortae were dissected and stained with oil red-O for morphometric analysis; blood/plasma was collected to determine markers of inflammation by cytokine array and cholesterol levels. *P. gingivalis*-induced bone loss in mandibles was assessed using micro-CT. *P. gingivalis* lipopolysaccharide stimulated nuclear factor-kappa B (NF-κB) activity was measured using a reporter gene (secreted alkaline phosphatase) assay in AP5055 treated or untreated RAW-Blue macrophages.

Results: Isolated aortae showed a significant decrease in lesion area in the AP5055 treated group as compared to the control group. Mechanistically, *in vitro* analysis demonstrated that AP5055 inhibited NF-κB activity. Cytokine array showed a decrease in the expression of pro-inflammatory cytokines and decreased levels of plasma cholesterol in AP5055 treated mice. Micro-CT measurements of bone loss were not significant between the two groups.

Conclusion: CD36 inhibitor AP5055 abrogates atherosclerotic lesion burden associated with periodontal disease, accompanied by a reduction in markers of inflammation. These experiments may support the development of drugs targeting CD36 for human disease.

Keywords: CD36 inhibitor, AP5055, periodontal disease, *Porphyromonas gingivalis*, atherosclerosis

1. Introduction

CD36 (also known as scavenger receptor B2) is a multifunctional transmembrane glycoprotein that was first identified on human platelet membranes as glycoprotein IV in the late 1970s (Okumura & Jamieson, 1976). This ~88Kda cell surface receptor has broad cellular distribution and is present on microvascular endothelial cells, monocytes/macrophages, megakaryocytes, hepatocytes, adipocytes, cardiac and skeletal myocytes and specialized epithelia of breast, kidney, and gut (Cho et al., 2005; Febbraio et al., 2001). The extensive cellular expression of CD36 contributes to a variety of normal and pathological processes. It is involved in the phagocytosis of apoptotic cells (Ren et al., 1995), endocytic uptake of modified lipoproteins (Endemann et al., 1993; Kunjathoor et al., 2002; Pearce et al., 1998) and has a role in lipid homeostasis, by binding to long-chain fatty acids with high affinity and promoting uptake into muscle and adipose tissue (Abumrad et al., 1993; Nassir et al., 2007). These functions of CD36 associate it with metabolic syndrome and cardiovascular disease (CVD).

Periodontal disease (PD) destroys the tissues supporting the tooth by an inflammatory process, which eventually can lead to tooth loss. It is the most common cause of tooth loss in adults. *Porphyromonas gingivalis (P. gingivalis)*, a gram-negative oral anaerobe is considered a keystone pathogen that, along with other pathogens, activates a destructive immune response directly and indirectly, by changing the oral microbiota (Leone et al., 2006). According to American Heart Association 2019 statistics, CVD remains one of the leading global causes of mortality. Atherosclerosis plays a major role in the underlying pathology of CVD. Several studies have suggested a strong link between PD and CVD, however, the mechanism(s) involved in a causal association and whether there is bidirectionality between the two remains incompletely understood (Bahekar et al., 2007; Lockhart et al., 2012).

Previous work showed that oral *P. gingivalis* infection, to induce PD, increased atherosclerosis lesion burden in a classic mouse model in the CVD field, the low-density lipoprotein receptor knockout (LDLR KO) (Brown et al., 2015). This increase was completely abrogated by genetic deletion of CD36, demonstrating an essential role for this protein (Brown et al., 2015). Mechanistically, the collaboration between CD36 and Toll-like receptor 2 (TLR-2) led to inflammasome activation by *P. gingivalis* lipopolysaccharide (*P. gingivalis* LPS), thereby resulting in interleukin-1 beta (IL-1β) synthesis, processing, and release (Brown et al., 2015). IL-1β is an important cytokine in the host response to bacteria in the oral cavity (Eskan et al., 2008) and also plays a significant role in the development of atherosclerosis (Tedgui & Mallat, 2006). This study showed that systemic IL-1β, as well as other pro-inflammatory cytokines, and oxidative stress, were increased to a greater extent in *P. gingivalis*-infected LDLR KO mice compared to CD36 KO/LDLR KO mice (Brown et al., 2015). This current study is a follow up to our work from Brown et al., showing that a portion of atherosclerosis in LDLR KO mice fed a high fat diet and infected with *P. gingivalis,* is CD36 dependent. This portion was the increase over the amount noted in LDLR KO mice fed a high fat diet alone. Important to this is the evidence that shows that, without an inflammatory component (*P. gingivalis),* high fat diet fed LDLR KO mice and CD36/LDLR double KO mice do not differ in atherosclerosis (Brown et al., 2015, Kennedy, et al., 2009).

In atherosclerotic lesion pathogenesis, CD36 binds and internalizes oxidatively modified low-density lipoproteins resulting in the formation of lipid-laden foam cells, an initiating event. This also activates signaling pathways, stimulating further vascular inflammation through secretion of cytokines and reactive oxygen/nitrogen species furthering lesion development (Febbraio et al., 2004; Guy et al., 2007).

The potential role for CD36 to serve as a therapeutic target has been inferred from studies using genetically engineered CD36 KO mice and small molecules to inhibit binding and uptake functions (Geloen et al., 2012). Work by the latter showed that one such inhibitor, AP5055, targets CD36 specifically, and can reduce atherosclerosis in the setting of hyperlipidemia and diabetes (LDLR KO/leptin-deficient mice) (Geloen et al., 2012).

The aim of this study is to determine if pharmacological inhibition of CD36, using the drug AP5055, can prevent the increase in *P. gingivalis*-mediated atherosclerosis in LDLR KO mice. In support of our hypothesis, we showed that a CD36 inhibitor, AP5055, was able to block the CD36-dependent component involved in atherosclerosis in the setting of *P. gingivalis* infection. These studies may support the use of inhibitors of CD36 in the prevention of PD-associated increased CVD.

2. MATERIALS AND METHODS

2.1. Animals

LDLR KO mice (10x backcrossed to C57Bl/6J; B6.129S7-Ldlr^{tm1Her}/J, strain #002207, The Jackson laboratory, Bar harbor, ME) were maintained and bred in a specific pathogen-free facility. Ethical approval for all animal procedures was procured beforehand from the Animal Care and Use Committee. Based on our previous study (Brown et al., 2015) and a power calculation analysis, 13 male LDLR KO mice per group were enrolled. We included an extra 3 mice per group because these are long studies and there was the potential for malocclusion, dermatitis, and complications due to multiple intraperitoneal (IP) injections/anesthesia. Mice were anesthetized IP with ketamine (100mg/kg) and xylazine (10mg/kg) for oral lavage procedures and euthanized by

pentobarbital overdose (200mg/kg IP) followed by bilateral thoracotomy, according to Canadian Council on Animal Care guidelines.

2.2. *Porphyromonas gingivalis* infection

P. gingivalis (*Porphyromonas gingivalis* ATCC ® 33277™ Manassas, VA) bacteria were grown for 72 hours on a blood agar plate under anaerobic conditions and then inoculated into Schaedler broth (Becton Dickinson, Franklin Lakes, NJ) containing vitamin K and hemin for 24-48 hours until the optical density (OD) of the culture reached 1.3 at 660nm (approximately 10^9 CFU/ml). Cultured *P. gingivalis* was resuspended in saline containing 2% carboxymethyl cellulose (as a thickener to promote adherence) before oral inoculation of mice. Six to eight-week-old LDLR KO male mice were orally lavaged with 200µl carboxymethyl cellulose containing ~10^{10} CFU/ml by using a micro brush at the gingival margin throughout the mouth on alternate days for two weeks. This procedure was performed in a level 2 biocontainment facility. Simultaneously, mice were fed a high fat, western diet (Harlan Teklad 88137, Envigo, Indianapolis, IN) ad libitum (21% fat, 0.15% cholesterol, no cholate) for 16 weeks to induce atherosclerosis. An important characteristic of the LDLR KO strain is that mice do not develop atherosclerosis unless they are fed a high fat diet (Ma et al., 2012; Kennedy et al., 2009).

AP5055 was synthesized by The Centre for Drug Research and Development (Vancouver, BC), at >95% purity, as determined by liquid chromatography-mass spectrometry. One-half of the mice received an IP injection of AP5055 (1mg/kg) in 1% dimethyl sulfoxide (DMSO), and the other half a daily IP injection of the vehicle alone (1% DMSO), every other day starting from day one and continuing for 16 weeks.

2.3. *En face* aortic morphometric analysis

Following euthanasia, the vasculature was perfused through the heart with 10 ml phosphate buffered saline (PBS) followed by 5 ml buffered formalin (Thermo Fisher Scientific, Waltham, MA). The entire aorta from the heart, including the subclavian, right and left carotid arteries, and extending 2-5 mm after bifurcation of the iliac, was dissected free of fat and post-fixed in buffered formalin for 24 hours at 4^{o}C and then stored in PBS at 4^{o}C. Aortae were stained with oil red-O, which identifies neutral lipids in plaque, to quantify lesion burden. After staining, the aortae were opened, laid flat, and digitally scanned. *En face* morphometry was performed in a blinded fashion, as previously described (Febbraio et al., 2000). Briefly, three independent measurements of lesion area (red pixels) were selected and averaged for each aorta, using Adobe Photoshop software. Similarly, the total aorta area was determined. The lesion area was expressed as mean percent $\pm$SE of total aortic area (Fig.1A-B).

2.4. Cell culture and secreted alkaline phosphatase (SEAP) promoter activity assay

RAW-Blue cells (InvivoGen, San Diego, CA) which are derived from murine RAW 264.7 macrophages, express TLR-2, CD36 and a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) secreted alkaline phosphatase (SEAP) reporter construct, were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin solution at 37^{o} C, 5% CO_2 in a humidified incubator. 10^5 cells/well were seeded in a 12-well plate and incubated overnight. Next day, when the cells reached 70-80% confluence, they were pre-treated with 125μg/ml or 150μg/ml of AP5055, or vehicle, for 30 minutes, followed by exposure to heat-killed *P. gingivalis* (a source of *P. gingivalis* LPS; 1:1000 dilution of 10^{10} CFU/ml, InvivoGen, San Diego, CA). After overnight incubation, cell culture supernatants were collected to measure SEAP activity with Quanti-blue (InvivoGen, San Diego, CA) detection medium using a spectrophotometer at OD 635 nm.

2.5. Cholesterol measurement

Mice were fasted overnight prior to sacrifice and blood collected into ethylenediaminetetraacetic acid (EDTA) containing syringes. Blood was centrifuged at 6000 rpm for 5 min and plasma aliquoted and frozen at -20^{o} C. Total plasma cholesterol was measured using a colorimetric cholesterol assay kit (Cell Biolabs Inc. San Diego, CA) as per the manufacturer's instructions. Levels in samples were determined by comparison against a curve constructed from a serially diluted cholesterol standard.

2.6. Cytokine array

The relative expression levels of 40 cytokines in plasma were compared between five pooled samples each from vehicle and AP5055 treated LDLR KO mice, using the Proteome Profiler Mouse Cytokine Array Panel A (R&D Systems, Minneapolis, MN). Membranes were washed and developed with enhanced chemiluminescence reagent and further visualized using an imaging system (ChemiDoc Gel Imaging System, Bio-Rad Laboratories, Hercules, CA). ImageLab software was used to measure pixel densities with a fixed selection of circular area and was placed over the grid-identified spot for each cytokine and chemokine. Each cytokine was measured in duplicate.

2.7. Micro-computed tomography (micro-CT) analysis

Mandibles were fixed in 10% buffered formalin for 24 hours at 4°C, followed by storage in PBS at 4°C prior to micro-CT (Milabs U-SPECT-II/CT, The Netherlands) analysis. Mice were imaged with a voxel size of 25 μm, and scan settings of 70 kVp, 114 μA, 0.5 mm AL filter, and integration time of 500 ms. A 3D construction was created using Avizo software (version 9.1, Thermo Fisher

Scientific, Waltham, MA). The distance from the cementoenamel junction to the alveolar bone crest in the first and second mandibular molars was measured using a landmark approach. The images were initially orientated on the coronal plane of the first molar to make the sagittal plane measurement (mesial and distal). The sagittal plane measurements were then taken through the long axis of the first molar of the root apex and the middle of the pulp chamber. The landmarks used were cementoenamel junction, alveolar bone crest, and root apex. In healthy mice/humans, the distance should be quite small, and increase with increasing bone resorption, brought on as a consequence of inflammation. Eight measurements were established: two in the sagittal plane and six in the coronal plane. The measurements were recorded in millimeters and compared to the control mice.

3.Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software. Results are presented as the standard error of the mean (SEM). Results were subjected to the D'Agostino and Pearson normality test. Those that passed normality were then assessed by two-tailed t-test or one-way ANOVA followed by Bonferroni's Multiple Comparison Test. If normality was not met, Mann-Whitney analysis was performed. Statistical significance was set at $p \leq 0.05$.

4. Results

4.1. AP5055 treatment in LDLR KO mice decreases atherosclerotic lesion burden

En face analysis revealed that AP5055 treated LDLR KO mice had decreased atherosclerosis lesion area compared with vehicle-treated mice (7.131 ± 0.732% *vs* 10.52 ± 0.661%, $p<0.01$) (Fig. 1A-B). This represents a reduction of 32% (Fig. 1C). These results are consistent with previous work showing *P. gingivalis*-infected CD36 KO/LDLR KO mice had significantly decreased lesion burden compared with *P. gingivalis*-infected LDLR KO mice. Thus, pharmacological inhibition

of CD36 similarly inhibited the increased atherosclerosis induced by *P. gingivalis* infection as genetic deletion.

4.2. AP5055 inhibits NF-κB activation in *P. gingivalis* LPS-stimulated murine macrophages

We examined the inhibitory effect of AP5055 on *P. gingivalis* LPS mediated activation of the NF-κB pathway using murine macrophage RAW-Blue cells, which stably express an NF-κB-SEAP reporter. NF-κB is the critical transcription factor downstream of TLR-2, responsible for inflammasome activation and cleavage of IL-1β into its active, secreted form. This cytokine plays a critical role in both PD and atherosclerosis. *In vitro* analysis of RAW-Blue cells showed increased production of SEAP in cells that received *P. gingivalis* LPS in comparison with the control group, in accordance with increased NF-κB activation (Fig. 2). Cells which were pre-treated with 125μg/ml or 150μg/ml AP5055 followed by *P. gingivalis* LPS stimulation exhibited a significant 42% and 55% decrease in SEAP production, ($p<0.001$, $p<0.0001$) respectively when compared to cells which were stimulated with *P. gingivalis* LPS and received vehicle alone (Fig. 2). These results suggest that AP5055 suppresses *P. gingivalis* LPS mediated transcriptional activation of NF-κB.

4.3. Cholesterol and weight measurements

Plasma samples were collected from both AP5055 and vehicle-treated LDLR KO mice infected with *P. gingivalis* at 16-weeks. The AP5055 treated mice group had significantly (618.5 ± 52.80 mg/dl SEM; $p<0.01$) decreased levels of plasma cholesterol as compared to the vehicle-treated group (867.2 ± 58.90 mg/dl SEM) (Fig. 3). No significant difference in weight was observed between the two groups.

4.4. Effect of AP5055 on cytokines/chemokines expression

Cytokine and chemokines are important mediators in the initiation and progression of atherosclerosis (Moss & Ramji, 2016). Circulating levels of pro-inflammatory cytokines in plasma, including tumor necrosis factor-alpha (TNF-α), interleukin-1 alpha (IL-1α) and monocyte chemoattractant protein-1 (MCP-1) were reduced in the AP5055 treated group relative to control (Fig. 4). In contrast, increased levels of interleukin-1 receptor antagonist (IL-1ra, 2.5fold) and B lymphocyte chemoattractant (BLC), were also observed. IL-1ra is a naturally occurring IL-1 inhibitor and has been shown to have anti-atherosclerotic roles: IL-1ra deficient mice develop more atherosclerosis (Isoda et al., 2004).

4.5. Alveolar Bone Loss

Alveolar bone loss in response to *P. gingivalis* oral lavage in both groups was evaluated using micro-CT. Results of the mandibular measurements showed no statistical difference regarding alveolar bone loss between the AP5055 treated and vehicle-treated groups (Fig. 5). These results show that both groups experienced equal levels of PD.

5. Discussion

The current study was designed to explore the antagonistic activity of a small molecule drug, AP5055, which specifically targets the macrophage scavenger receptor CD36. Our major finding was that targeting CD36 with AP5055 abrogates the increased atherosclerotic lesion burden associated with PD. Previous work using the oral lavage method of *P.gingivalis* infection in the LDLR KO mouse suggested that the inflammation in the oral cavity increased systemic inflammation to create CD36 ligands as a result of oxidative modification of LDL, and this drove the increase in lesion burden (Brown et al., 2015) .These results further support that mechanism: AP5055 treatment was accompanied by a decrease in pro-inflammatory, pro-atherosclerotic cytokines/chemokines, thereby reducing inflammation, as shown by our cytokine array results.

There are significant studies in the literature, including our own, showing that *P. gingivalis* increases atherosclerosis in mouse models (Brown et al., 2015). No control group without *P. gingivalis* infection was included in this study as this has been shown in our previous study and in accordance with recommendations to reduce animal usage. The current study and Brown et al., used a low cholesterol diet and *P. gingivalis* as the inflammatory stimulant, to mechanistically link *P. gingivalis* infection to LDL modification through oxidative stress, and CD36 (Kennedy et al., 2009). In our study, AP5055, was shown to block the CD36-dependent component involved in atherosclerosis in the setting of *P. gingivalis* infection.

In a study by Geloen et al., AP5055 was shown to reduce postprandial hyperlipidemia and the progression of atherosclerosis in LDLR KO/leptin-deficient mice. These mice have a metabolic phenotype that results in obesity and insulin resistance, in addition to hyperlipidemia. Mechanistically, this group showed that AP5055 inhibited fatty acid and oxidized low density lipoprotein uptake in CD36-expressing HEK293 cells, resulting in less severe insulin resistance and better lipoprotein and metabolic profiles. Accumulation of lipid vesicles in these cells was also inhibited by this anti-CD36 compound. A decrease in plasma cholesterol levels, similar to what we observed, was noted in that study.

The 32% reduction in lesion burden as a result of AP5055 treatment in our study was similar to the 46% decrease observed in LDLR KO/leptin-deficient mice (Geloen et al., 2012) and the 52% decrease reported in *P. gingivalis*-infected male CD36 KO/LDLR KO mice compared to *P. gingivalis*-infected LDLR KO mice (Brown et al., 2015). The reason there may have been slightly more inhibition in the latter study, which had a similar design to ours, is that genetic ablation of CD36 inhibits all CD36-dependent functions at all times, whereas drug inhibition may wane between doses, and AP5055 may not inhibit all CD36-dependent pro-atherosclerotic mechanisms.

A difference between our study and the Geloen study is that we gave AP5055 every other day instead of every day, to reduce stress on the mice.

The inoculation procedure was adapted from the method of Lalla et al., (Lalla et al., 2003). In that study, mice were given 15 oral inoculations of *P. gingivalis* over a 3-week period. In Brown et al., we modified this procedure to 14 inoculations over a 2-week period and showed that this successfully induced bone loss and accordingly that method was used in our current study. The 16-week time point to allow atherosclerosis development was selected because this is a common time point used in the literature for enface analysis of the aorta (Guy et al; 2007).

The importance of CD36 in the development of atherosclerosis is well established (Febbraio et al., 2001; Zhao et al., 2018) particularly with regard to macrophage foam cell formation, the initiating lesion in plaque formation. Both *in vivo* and *in vitro* studies have shown a significant decrease in uptake of modified forms of LDL and foam cell formation using cells and strains derived from CD36 KO mice (Febbraio et al., 1999; Podrez et al., 2000; Rahaman et al., 2006)**.** Further work implicated CD36 in cell signaling that contributes to the chronic inflammatory, pro-atherosclerotic condition (Silverstein et al, 2010; Zhao et al., 2018).

Our *in vitro* analysis, using the NF-κB reporter macrophage cell line, RAW-Blue cells, demonstrated significant inhibition of *P. gingivalis* LPS mediated NF-κB activation by AP5055. We observed a reduction in SEAP in the presence of AP5055 leading to less activation of NFκB which results in less IL-1β generation. This is a novel and significant finding because it is known that *P. gingivalis* LPS activation of the NF-κB signaling pathway is essential for inflammasome activation and processing and release of IL-1β, an essential cytokine in atherosclerosis (Brown et al., 2015; Ramji & Davies, 2015). While lesion burden was reduced, we observed no differences in bone loss, suggesting that AP5055 had little effect in the oral cavity. In previous work, we

observed that the genetic ablation of CD36 also impacted bone loss (Brown et al., 2015). It may be that, in this study, the dose of the drug was inadequate, or that the CD36-dependent mechanism involved in the bone loss was not inhibited by AP5055.

Conclusion

Overall, our work demonstrates that targeting CD36 using a small molecule inhibitor reduces atherosclerosis lesion burden and systemic inflammation in a mouse model. These studies suggest that targeting CD36 may be a feasible strategy in reducing the additional risk of CVD by factors that contribute to systemic inflammation, such as periodontal disease.

CRediT authorship contribution statement

MF and UR contributed to the concept and design of the study. UR, MF and MA were involved in sample collection. RC contributed protocols for preparation of samples for micro-CT and for micro CT analysis. RC also contributed expertise in analysis and interpretation of the micro-CT data. MA performed the micro-CT scans. AF was involved in analysis of micro-CT scans. MS helped with the SEAP assay and figures. UR and MF interpreted the data and drafted the manuscript, with input from RC. UR, MF and RC have ensured that the accuracy and integrity of the work have been appropriately investigated. All authors have given final approval of the version to be published.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Figure Legends

Figure 1

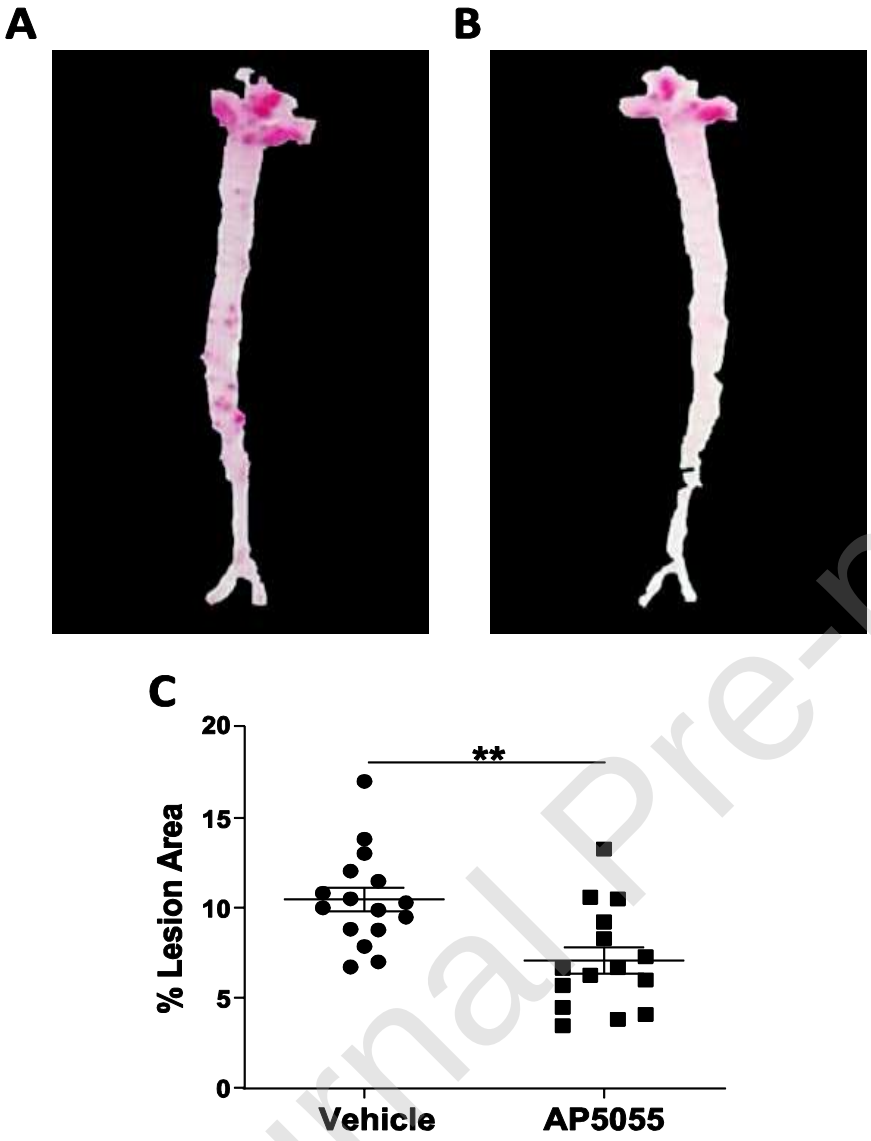

Figure 1: Effect of CD36 inhibitor on lesion burden in the aorta. A, B. Representative aortae from high fat Western diet fed LDLR KO mice orally infected with *P gingivalis* and A, injected with vehicle or B, with AP5055. Oil red-O positive areas indicate atherosclerotic plaque. C. Quantitative analysis of lesion area as % of total aorta. There was a significant difference between the vehicle and AP5055-treated group. Two-tailed t- test (**$p \leq 0.01$). n=15 AP5055 group, n=16 vehicle group

Figure 2

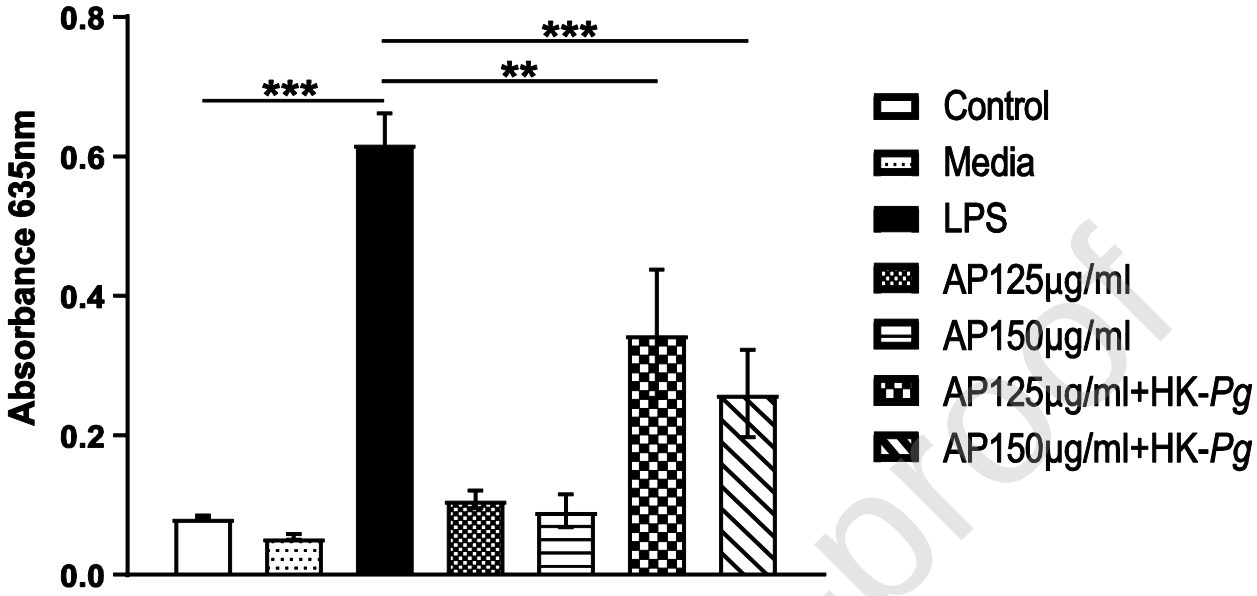

Figure 2: Effect of AP5055 on *P. gingivalis*-mediated activation of NF-κB in murine macrophages. NF-κB activation measured by reporter gene assay (SEAP activity). RAW-Blue cells were incubated with heat killed *P. gingivalis (*HK-*Pg)* after 30 minutes pre-treatment with different doses of AP5055. SEAP activity as a marker of NF-κB activation was detected using a colorimetric substrate at OD 635nm. One-way ANOVA, (*$p \leq 0.001$ ***$p \leq 0.0001$). Bonferroni's Multiple Comparison test. n=4 replicates per group.

Figure 3

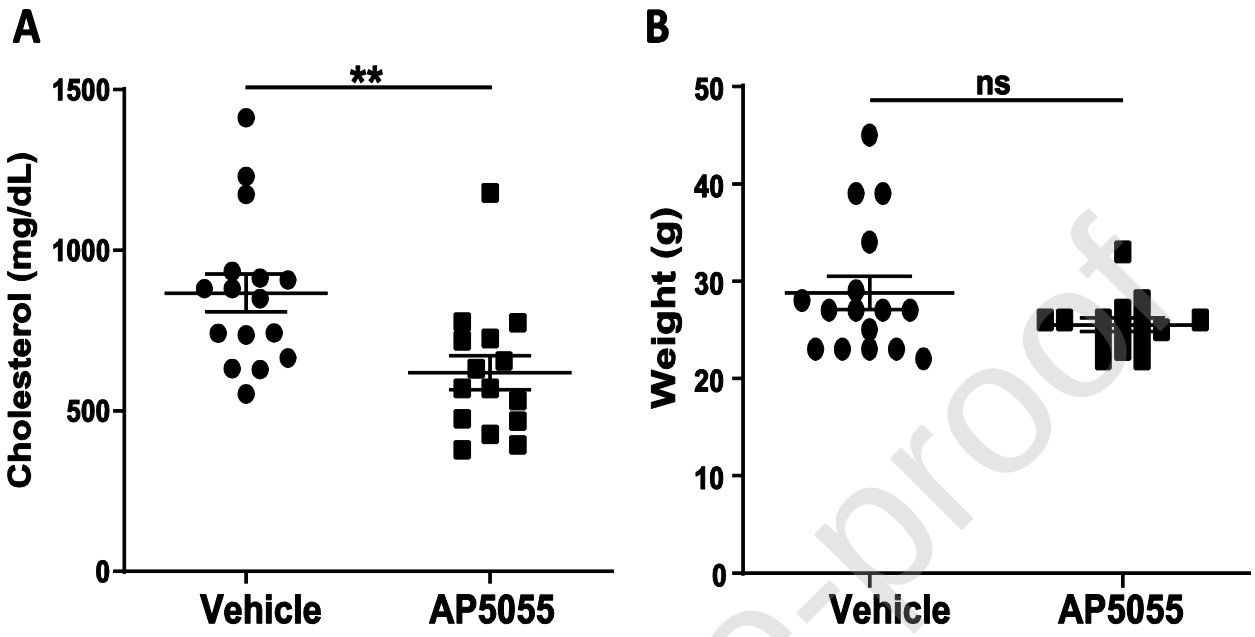

Figure 3: Effect of CD36 inhibitor on plasma cholesterol levels and weight. Cholesterol and weight were measured at 16 weeks in vehicle and AP5055-treated mice. A) Quantitative analysis of cholesterol concentration in plasma samples. There was a significant difference between the vehicle and AP5055-treated group. Two-tailed t- test (**$p \leq 0.01$). B. There was no significant difference in weight between the groups (ns=not significant). n=15 AP5055 group, n=16 vehicle group

Figure 4

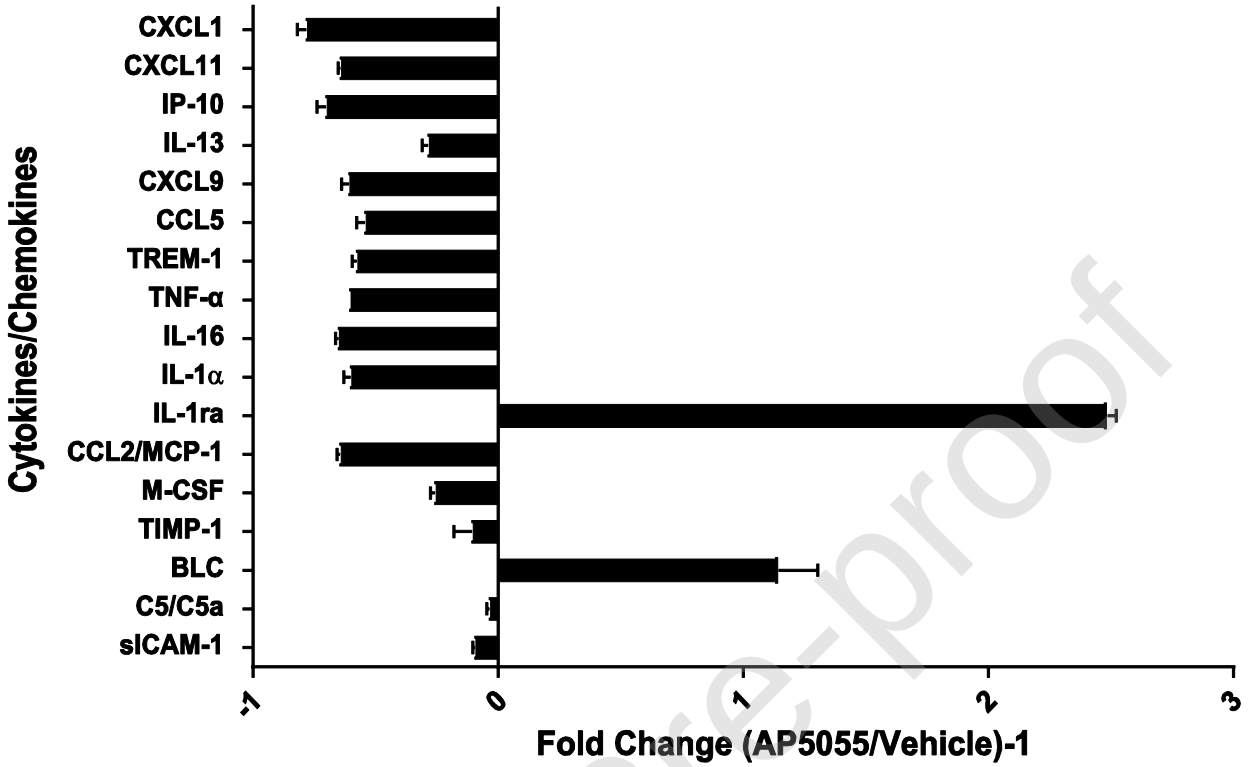

Figure 4: Effect of AP5055 on the production of inflammatory mediators. The relative concentrations of inflammatory cytokines/chemokines in 16-week plasma samples (5 mice plasma pooled/group) were measured in duplicate using a cytokine array. Densitometric analysis was performed to calculate relative expression levels. The bar-graph represents the fold change in AP5055-treated mice compared with vehicle–treated mice.

Figure 5

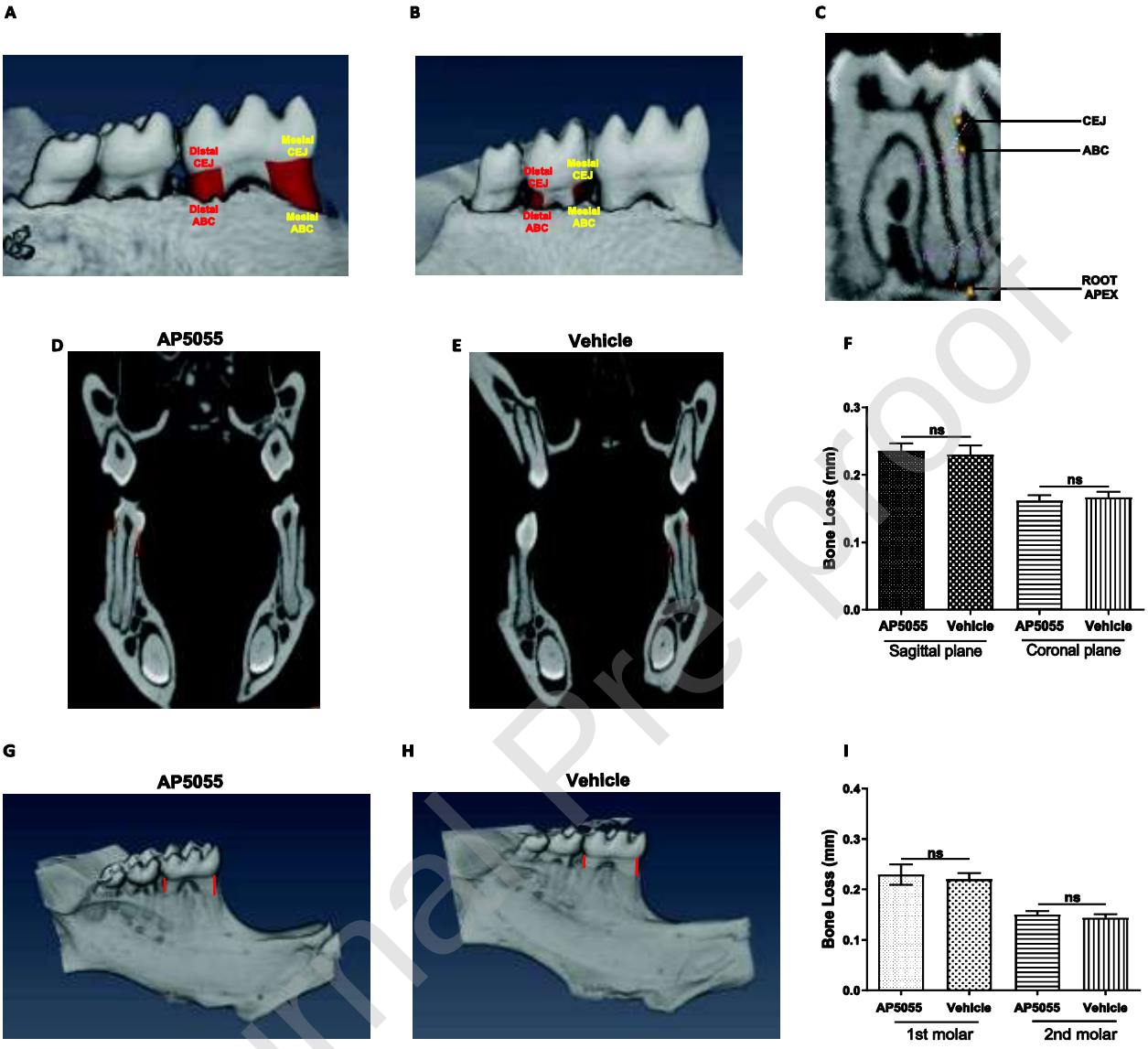

Figure 5: Effect of AP5055 on alveolar bone loss. Micro-CT was used to measure bone loss in the 1st and 2nd molars of the mandible. A) Three-dimensional image illustrating mesial and distal of the first molar from a sagittal view. B) Three-dimensional image illustrating mesial and distal of the second molar from a sagittal view. C) Sagittal view of landmarks used for measurements in the sagittal view (cementoenamel junction (CEJ), alveolar bone crest (ABC), root apex). Representative micro-CT images of coronal plane: D) AP5055-treated mice; E) Vehicle-treated

mice. The red line indicates the distance from the cementoenamel junction to the alveolar bone crest. F) quantitative analysis of bone loss of coronal and sagittal plane. Sagittal plane: G) AP5055-treated mice; H) Vehicle-treated mice. The red line indicates the distance from the cementoenamel junction to the alveolar bone crest. I) Bar-graph represents quantitative analysis of bone loss of 1^{st} and 2^{nd} molars respectively. There was no significant difference in bone loss between the groups (ns=not significant).