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The Peridontal Pathogen, *Porphyromonas gingivalis,* and the Participatory Role of Caspase-1 in the Progression of Atherosclerosis

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THE PERIODONTAL PATHOGEN, *Porphyromonas gingivalis,* AND THE PARTICIPATORY ROLE OF CASPASE-1 IN THE PROGRESSION OF ATHEROSCLEROSIS

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ABSTRACT

Porphyromonas gingivalis, a key pathogen in periodontal disease, promotes atherosclerosis in apolipoprotein E null (*apoe*$^{-/-}$) mice, a well characterized mouse model of the disease. Previous studies have provided indirect evidence that *P gingivalis* is capable of activating the enzyme caspase-1 *in vitro*, while preliminary studies conducted by our laboratory have outlined a role for caspase-1 in atherosclerosis in *apoe*$^{-/-}$ mice. The present study provides direct evidence that *P gingivalis* is capable of activating caspase-1, and that *P gingivalis* is capable of gaining access to, and localizing at, sites of lesion development following an oral challenge. Oral infection with *P gingivalis* exacerbated the development of atherosclerosis in the aortic root of male mice competent for caspase-1 as compared to those deficient in the enzyme. Infected animals also displayed evidence of alveolar bone loss, a hallmark of periodontal disease. Our findings reveal that oral infection with *P gingivalis* accelerates atherosclerotic lesion development in male *apoe*$^{-/-}$ mice through the activation of caspase-1.

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LIST OF ABBREVIATIONS

ABC	alveolar bone crest
apo	apolipoprotein
apoE	apolipoprotein E
ASC	apoptosis-associated speck-like protein containing a CARD
Asp	aspartic acid
ATCC	American Type Culture Collection
CARD	caspase activation and recruitment domain
Casp1	caspase-1
CE	cholesteryl ester
CED	cell-death-defective
CEJ	cemento-enamel junction
CFU	colony forming unit
CO_2	carbon dioxide
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbant assay
EM	electron microscopy
ER	endoplasmic reticulum
FBS	fetal bovine serum
FC	free cholesterol
FimA	fimbriae
FPLC	fast performance liquid chromatography
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidise
ICE	interleukin-1β converting enzyme; caspase-1
IDL	intermediate-density lipoprotein
IDV	integrated density value
IgG	immunoglobulinG
IGIF	interferon-γ inducing factor; IL-18
IL-18	interleukin-18

IL-1β	interleukin-1β
IMT	intima-medial thickness
Ipaf	ICE protease activating factor
LDL	low-density lipoprotein
LDLr	low-density lipoprotein receptor
LPS	lipopolysaccharide
LRR	leucine rich repeat
MOI	multiplicity of infection
NLR	nucleotide-binding oligomerization domain-like receptor
NOD	nucleotide binding oligomerization domain
OCT	optimum cutting temperature
oxLDL	oxidized low-density lipoprotein
PAMP	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PG	*Porphyromonas gingivalis*
PRR	pattern recognition receptor
PVDF	polyvinylidene difluoride
RIPA	radioimmunoprecipitation
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulfate
SMC	smooth muscle cell
SR-A	scavenger receptor A
TLR	toll-like receptor
VLDL	very low-density lipoprotein

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CHAPTER 1 : INTRODUCTION

Atherosclerosis is one of the major causes of morbidity and mortality in the Western world. Although traditional risk factors clearly contribute to the pathogenesis of atherosclerosis, they do not account for all disease cases. To this end, several putative risk factors for atherosclerosis have been proposed, with periodontal disease emerging as a candidate risk factor. *Porphyromonas gingivalis*, a key pathogen in periodontal disease, has been shown to promote the development of atherosclerotic lesions in a well characterized mouse model of atherosclerosis - the apolipoprotein E null (*apoe*$^{-/-}$) mouse. Previous studies have provided indirect evidence that *P gingivalis* is capable of activating the enzyme caspase-1 *in vitro*. Interestingly, preliminary studies conducted by our laboratory have outlined a role for caspase-1 in the development of atherosclerosis in *apoe*$^{-/-}$ mice. The present study examines whether infection by a periodontal pathogen, *Porphyromonas gingivalis*, promotes atherosclerotic lesion development in *apoe*$^{-/-}$ mice, through the activation of the pro-inflammatory enzyme caspase-1.

CHAPTER 2 : BACKGROUND

2.1 Cardiovascular disease

Cardiovascular disease resulting from atherosclerosis is one of the major causes of morbidity and mortality in the Western world and is the foremost underlying cause of myocardial infarction, stroke and peripheral artery disease (Lucas and Greaves, 2001;Whitman, 2004). Atherosclerosis is a chronic disease that affects the large and medium sized arteries of the circulatory system. It is characterized by the thickening and hardening of the blood vessel walls, and results from the excessive accumulation of lipids, inflammatory cells and cellular debris within the inner lining of the artery, forming what is called a lesion (Ross, 1999). Atherosclerosis is therefore considered an unusual form of chronic inflammation that occurs within the arterial wall (Lucas and Greaves, 2001;Ross, 1999). With the general acceptance of atherosclerosis as a chronic inflammatory disease there has been a rejuvenation of efforts to examine the role played by infectious agents in the pathogenesis of atherosclerosis.

2.2 Atherosclerosis

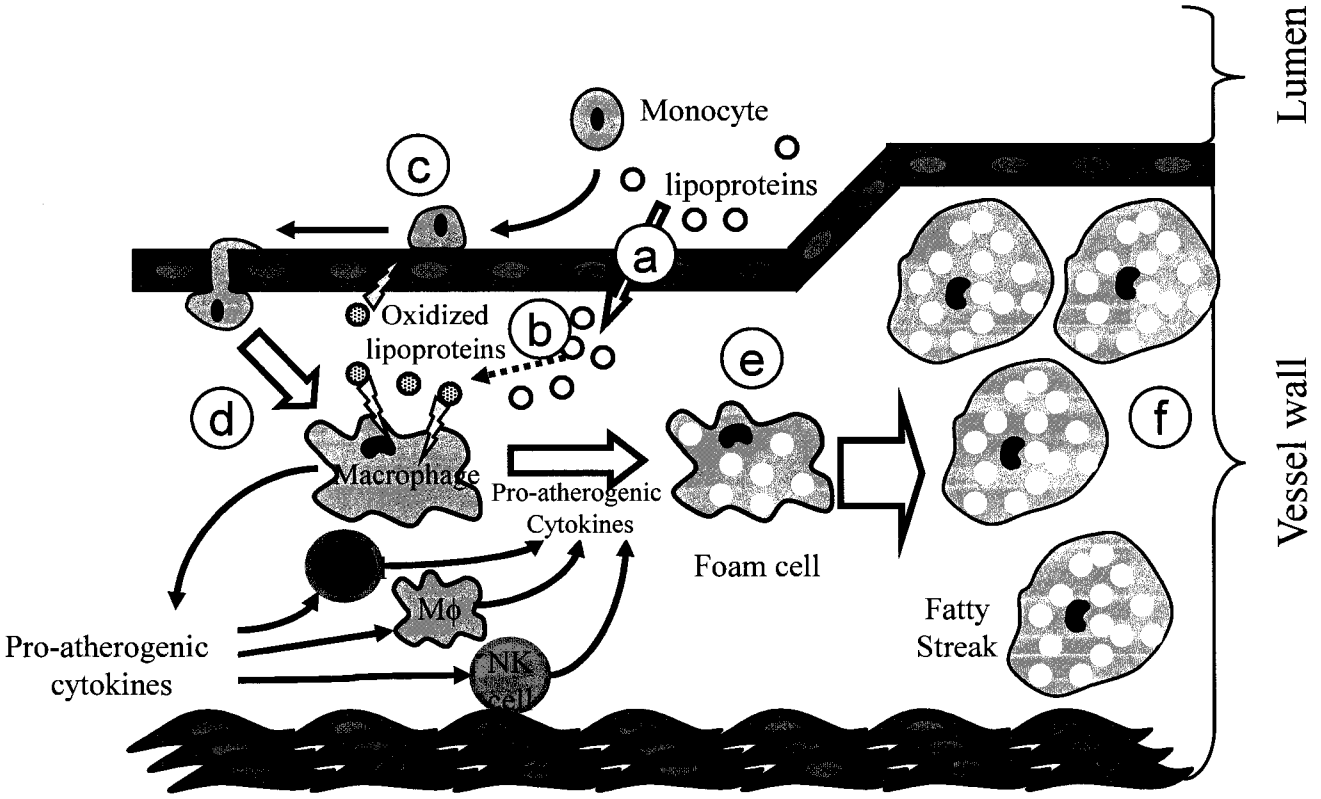
2.2.1 Working model of atherosclerosis

Atherosclerotic lesions form through a dynamic process which progresses through a series of three preliminary stages: the fatty streak, the fibrous plaque, and the complicated lesion (Whitman, 2004). Fatty streaks form from an accumulation of lipoproteins, macrophages, and lymphocytes within the innermost layer of the arterial vessel wall, called the tunica intima (Lucas and Greaves, 2001) (**Figure 1**). As lipoproteins enter the tunica intima they become trapped and modified via oxidation

(Steinberg and Witztum, 1990) to a form that is chemotactic for monocytes (Quinn *et al.*, 1987). Monocytes migrate from the bloodstream into the tunica intima of the vessel wall where they differentiate into macrophages and begin to clear the modified oxidized lipoproteins (Whitman, 2004). The lipid components of these modified lipoproteins accumulate in an unregulated manner within the intimal-associated macrophages giving them a morphological "foamy" appearance, hence these lipid-filled macrophages are called foam cells (Whitman, 2004) (foam cells are discussed in further detail in Chapter 2.2.2: "Foam cell formation" on page 5). A build-up of these foam cells form the fatty streak, which is the earliest detectible atherosclerotic lesion (Stary *et al.*, 1994). Macrophages are therefore considered instrumental in the formation and progression of atherosclerotic lesions. Fatty streaks are not occlusive to blood flow and are not clinically relevant, however they may progress to more complex lesions that can give rise to chronic symptoms (Vance and Vance, 2002). Over time the macrophage-derived foam cells die and contribute their intracellular lipids to the necrotic core of the growing lesion (Lusis, 2000). More advanced lesions result from the continuous recruitment of macrophages within the intima, as well as the migration and proliferation of smooth muscle cells (SMCs) to the sites of lesion development which secrete large amounts of collagen and other matrix proteins (Lucas and Greaves, 2001). With the secretion of these elements by SMCs, fibrous plaques develop which become occlusive to blood flow as they increase in size (Lusis, 2000). These events give rise to complex fibrous lesions consisting of foam cells and smooth muscle cells covered by a fibrous cap (Lusis, 2000). Initially lesions grow toward the adventitia of the vessel, but a critical point is reached where they begin to expand and subsequently encroach into the lumen of the artery,

Figure 1: Working model of atherosclerosis.

Proposed mechanism by which specific components of the immune system can promote the formation of early atherosclerotic lesions via the enhancement of macrophage-derived foam cell formation. Various components play a role in the development of the fatty streak, which is the first pathological change in atherosclerosis within the inner most layer of the blood vessel wall, the tunica intima. **(A)** The first step in the development of the lesion is the accumulation of plasma derived lipoproteins within the tunica intima where these lipoproteins are modified by oxidation **(B).** **(C)** The presence of oxidized lipoproteins initiates the migration of monocytes from the lumen to the tunica intima. **(D)** Once inside the vessel wall, monocytes undergo differentiation into macrophages where they begin to clear away the oxidized lipoproteins. **(E)** As the macrophages accumulate lipoproteins, they become engorged with lipids, forming a foam cell. **(F)** An accumulation of foam cells forms a fatty streak which is the earliest detectible atherosclerotic lesion.


Lumen
Vessel wall
Monocyte
lipoproteins
c
a
b
Oxidized
lipoproteins
d
e
f
Macrophage
Pro-atherogenic
Cytokines
Foam cell
Fatty
Streak
Mϕ
NK
cell
Pro-atherogenic
cytokines

disrupting blood flow (Lusis, 2000). Over time, lesion formation results in the development of large, structurally unstable lesions that are prone to rupture, thrombus formation, occlusion of the vessel, and death of the tissue distal to the area of lesion formation (Whitman, 2004).

2.2.2 Foam cell formation

Foam cells are formed through the excessive accumulation of lipids within the macrophage. The majority of low-density lipoprotein (LDL)-derived cholesterol within the cell exists in the esterified form. Cholesteryl esters (CE) are hydrolyzed in the late endosomal/lysosomal compartments by lysosomal acid lipase, and the resulting free cholesterol (FC) is then transported to other cellular sites (Vainio and Ikonen, 2003). Cholesterol is normally transported from their lysosomal compartments to the plasma membrane where it is accessible to the endoplasmic reticulum (ER) and can undergo re-esterification (Vainio and Ikonen, 2003). Foam cells accumulate a large amount of CE that is stored within the cell as cytosolic lipid droplets, which is accompanied by rapidly increasing amounts of cellular FC (Vainio and Ikonen, 2003).

Cholesterol is essential for many cellular processes and is an essential component of mammalian cells. In macrophages, cholesterol derived from LDL enters the cell *via* receptor-mediated pathways (Vainio and Ikonen, 2003). These receptors recognize and respond differently to native and modified forms of lipoproteins. Native LDL is taken up by the macrophage through LDLr-mediated endocytosis. Transcription of the LDLr gene is regulated by the amount of cholesterol within the cell, so that when cholesterol is abundant, LDLr expression decreases (Vainio and Ikonen, 2003). Since the uptake of cholesterol *via* the LDLr is downregulated by increased amounts of cholesterol, this route

of cholesterol uptake does not lead to excessive lipid accumulation within the cell. Therefore macrophage uptake of native LDL does not contribute to the formation of foam cells during the early stages of atherosclerosis.

During the formation of atherosclerotic plaques, lipoproteins enter the tunica intima of the blood vessel wall where they are modified by oxidation. Uptake of this modified form of LDL (oxLDL) is not under the same homeostatic regulation as the uptake of native LDL (Vainio and Ikonen, 2003). Modified LDL is not taken up by the macrophage through LDLr-mediated endocytosis, but rather is recognized by a different class of macrophage surface receptors termed the scavenger receptors (Vainio and Ikonen, 2003). It has been demonstrated that the scavenger receptors SR-A and CD-36 on the surface of the macrophage recognize and internalize oxLDL (Vainio and Ikonen, 2003). In this cholesterol uptake pathway, oxLDL stimulates its own uptake by upregulating the expression of surface receptors. The result is a rapid accumulation of oxLDL within the macrophage thereby generating the foam cell.

2.3 Mouse models for atherosclerosis

Atherosclerotic events in humans are not usually detected until the advanced stages, long after they become clinically relevant. Thus, studying human atherosclerotic events in their later stages does not provide insight into how atherosclerotic lesions develop and progress in their earliest stages. To this end, the mouse has become a powerful tool in investigating biology and molecular pathways involved in the formation atherosclerotic lesions at their earliest stages.

2.3.1 Cholesterol transport

Cholesterol is essential in all mammalian cells for proper cellular functions. It is either synthesized in the endoplasmic reticulum, or derived from the diet where it circulates through the body in the bloodstream (Tortora and Grabowski, 2003). Cholesterol is insoluble in blood and therefore has to be bound to water-soluble proteins, known as lipoproteins, to be transported in the circulatory system throughout the body (Vance and Vance, 2002). Lipoproteins rely on apolipoproteins (apos) associated with their surface to accurately target lipoproteins to sites of metabolism and removal (Vance and Vance, 2002). Apos function by mediating the interaction of lipoproteins with enzymes, transfer proteins and cell surface receptors (Tortora and Grabowski, 2003).

2.3.2 Apolipoprotein E

Apolipoprotein E (apoE) was first identified in 1973 as a member of the evolutionary conserved apolipoprotein gene family which includes apoA-I, apoA-II, apoA-IV, apoC-II, and the apoC-I pseudogene (Plump and Breslow, 1995). Its function and importance are thoroughly outlined in a review by Plump and Breslow (Plump and Breslow, 1995). ApoE associates with the surface of plasma lipoprotein particles and mediates the interaction with cell surface receptors. ApoE is the primary ligand for low-density lipoprotein (LDL) receptor-mediated removal of lipoprotein remnants from the circulation (Plump and Breslow, 1995). Although the LDL receptor is also capable of binding lipoprotein particles containing another physiological ligand, apoB100, it is apoE that is the most potent known physiological ligand for the LDL receptor (Plump and Breslow, 1995). Lipoprotein particles containing apoB100 bind the LDL receptor at only 5% affinity of those that contain apoE (Plump and Breslow, 1995). ApoE is recognized

by 2 distinct receptors: the LDL receptor (LDLr) and the chylomicron remnant receptor. Larger apoE-associated lipoprotein particles, such as very-low density lipoprotein (VLDL) and β-VLDL are cleared by the LDLr, while smaller particles, such as chylomicron remnants and intermediate density lipoproteins (IDL) can be cleared by either the LDL receptor or the chylomicron remnant receptor (Plump and Breslow, 1995). ApoE is therefore essential for the removal of lipoproteins and their cargo from the circulation via the LDLr.

2.3.3 The *apoe*$^{-/-}$ mouse model

Mice have been invaluable in giving insight into many human disease processes. This model possesses several advantages, such as the ability to perform genetic manipulations using transgenic and gene-targeting technology, they are easy to breed, have a short generation time, and inbred strains are readily available (Plump and Breslow, 1995). Unfortunately, wild-type mice are naturally resistant to the development of atherosclerotic lesions. In order to make mice susceptible to atherosclerosis, the lipid metabolism of the mouse must be altered. One of the ways by which this is achieved is through the generation of a mouse model that is deficient in apoE (*apoe*$^{-/-}$). The ability of the *apoe*$^{-/-}$ mouse model to clear plasma lipoproteins is impaired, causing the mice to become hyperlipidemic (Plump and Breslow, 1995). When fed a low-fat diet the *apoe*$^{-/-}$ mice have plasma cholesterol levels of 500 mg/dL, whereas mice competent for apoE have plasma cholesterol levels closer to 75 mg/dL (Plump and Breslow, 1995). The massive elevation of plasma cholesterol levels in the *apoe*$^{-/-}$ mouse model is primarily due to an increase in the VLDL, and VLDL remnant (IDL) cholesterol fractions (Plump and Breslow, 1995).

2.3.4 Comparison between atherosclerosis in humans and mice

Atherosclerosis is a complex disease which begins early in childhood in humans, but does not become clinically relevant until later in life (Whitman, 2004). As lesions become clinically relevant, they pass through a series of six predicted stages, whose development is based on morphological criteria (Whitman, 2004). These morphological criteria have been thoroughly reviewed by Dr. Stewart Whitman (Whitman, 2004). The morphological features of early stages lesions in *apoe*$^{-/-}$ mice are very similar to those seen in humans. As with lesion development in humans, in mice these early lesions consist of an accumulation of lipoproteins within the tunica intima of the vessel wall and a gathering of monocyte-derived macrophages that originate from the blood stream (these lesions are classified as stages I-III) (Whitman, 2004). An accumulation of extracellular lipid, known as the lipid core, begins to occupy an extensive area of the tunica intima as the lesions become more advanced (stage IV lesions). When the tunica intimal area on the luminal side of the lipid core begins to accumulate fibrous tissue, a cap is formed over the entire lesion (stage V lesions). Lesions become more complicated as they develop necrotic cores (stage VI) and are capable of developing haematomas and/or thrombi. It is these more advanced stage lesions that become clinically relevant in humans as morbidity and mortality is largely due to a disruption of these lesions. Whether or not these advanced stage lesions actually develop in the *apoe*$^{-/-}$ mouse model is a matter of debate, however the progression of early stage lesions in mice mirrors the development of atherosclerotic lesion formation in humans.

2.3.5 Quantification of atherosclerosis in mice

Although there a number of methods for the quantification of atherosclerosis in mice, the three most commonly used are: (a) cross-sections of the aortic root and ascending aorta, (b) an *en face* preparation of the aortic tree covering the arch, thoracic and abdominal regions of the aorta opened longitudinally to expose the area of lesion covering the luminal surface, and (c) serial sections of the proximal 1 mm of the branchiocephalic trunk which is the first major branch off of the aorta at the region of the aortic arch (Whitman, 2004).

2.4 Risk factors for atherosclerosis

A variety of risk factors are known to be associated with atherosclerosis and the pathogenesis of cardiovascular disease. These include traditional genetic and lifestyle risk factors such as elevated low density lipoprotein (LDL) cholesterol levels, hypertension, hypoglycaemia, stress, smoking, and obesity (Wilson *et al.*, 1998).

Several studies have been published concerning the relationship between conventional risk factors and atherosclerosis, where conventional risk factors have been shown to account for anywhere between 50-70% of the disease in the general population (Haynes and Stanford, 2003). Traditionally these studies make use of single-risk or single-population studies where one risk factor is examined in one specific population. Although these studies have been instrumental in identifying risk factors for atherosclerosis, some data suggest that risk factors for atherosclerosis may vary between populations and researchers are therefore uncertain as to what extent these findings apply worldwide (Yusuf *et al.*, 2004). In 2004, the INTERHEART study was published as a

large, international, standardized, case-control study which examined the effect of potential modifiable risk factors associated with myocardial infarction in the global population (Yusuf *et al.*, 2004). This study was the first of its kind which focused on examining a large number of individuals from all regions of the world and multiple ethnic groups, making it one of the most broadly applicable studies to be published to date. The objective was to determine the association between nine measurable risk factors (smoking, lipids, self-reported hypertension or diabetes, obesity, diet, physical activity, alcohol consumption, and psychosocial factors) and acute myocardial infarction, and to fully establish whether this association varies by geographic region, ethnic origin, sex, or age (Yusuf *et al.*, 2004). Subjects were recruited from 262 centres from 52 countries in Asia, Europe, the Middle East, Africa, Australia, North America, and South America. For the purposes of the study 15,152 cases and 14,820 controls were enrolled following admittance to the coronary care unit (or equivalent cardiology ward) within 24 hours of acute myocardial symptom on-set, and if they had characteristic symptoms indicative of a new acute myocardial infarction. This ground-breaking study reported that abnormal lipids, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, consumption of fruits, vegetables, and alcohol, and regular physical activity account for approximately 90% of the risk for myocardial infarction worldwide.

While INTERHEART and other population-based studies clearly outline the contribution of traditional risk factors to cardiovascular disease, it has become clear that these traditional risk factors do not account for all disease cases. To this end, several putative risk factors for atherosclerosis have been proposed, with periodontal disease emerging as a candidate risk factor.

2.5 Periodontal disease

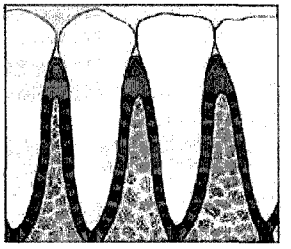
Periodontal disease encompasses a group of infections that affect the supporting tissues of the teeth (**Figure 2**), ranging in severity from mild inflammation of the gingival tissue to chronic destruction of the periodontal tissues (gingival tissue, periodontal ligament and alveolar bone) (Lamont and Jenkinson, 1998). It is a chronic infection caused by the bacteria of dental plaque, which begins as inflammation of the soft tissues (gingivitis) and can progress to periodontitis. In the mildest forms of gingivitis the gums become red, swollen, and can bleed easily. This form of gum disease can be reversed with brushing and flossing, and does not include any loss of bone or tissue that holds the teeth in place. If left untreated, gingivitis can progress to periodontitis, where gums begin to pull away from the teeth and form pockets around the teeth that become infected. As a result of bacteria spreading and beginning to grow below the gum line, bacterial toxins and the body's immune response can begin to break down the bone and connective tissues holding teeth in place (Tortora and Grabowski, 2003). The progression of periodontal disease leads to the complete destruction of the alveolar bone and the connective tissue, eventually leading to tooth loss. Periodontal disease is more common in people who smoke (Molloy *et al.*, 2004), are obese (Al-Zahrani *et al.*, 2003), and have diabetes (Lalla *et al.*, 2004;Molloy *et al.*, 2004). This disease affects 75% of adults in the United States with 20-30% of them having severe forms (Papapanou, 1999).

The oral cavity houses a wide variety of bacterial species, with over 300 different bacterial species having been isolated from human subgingival plaque samples (Marsh, 1989;Wang and Ohura, 2002), where anywhere from 1000 to 1 billion bacteria have been reported to live on each tooth surface (Duncan *et al.*, 2002). However, only some of

Figure 2: Progression of periodontal disease.

Periodontal disease is a chronic infection caused by the bacteria of dental plaque that encompasses a group of infections that affect the supporting tissues of the teeth, ranging in severity from gingivitis to periodontitis. **(A)** In normal, healthy individuals gums anchor the teeth firmly in place. **(B)** Gingivitis is caused by irritation of the gingival tissue by dental plaque, which makes the gums tender and red, and causes them to bleed easily. **(C)** In periodontitis, dental plaque accumulates and hardens, the gums recede from the teeth, and deep pockets form between the teeth and the gums. **(D)** As the disease progresses the gums recede further and bacterial colonization of the deep pockets between the gums and the teeth causes destruction of the connective tissue attachment and the alveolar bone, eventually leading to tooth loss. (Pictures by American Academy of Periodontology)

A.



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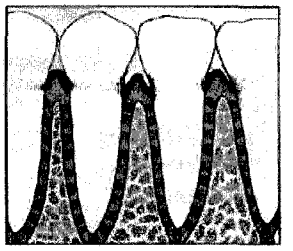

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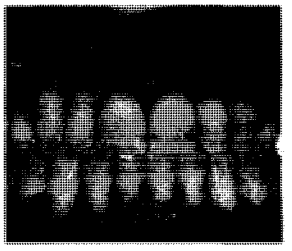
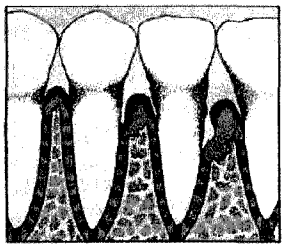
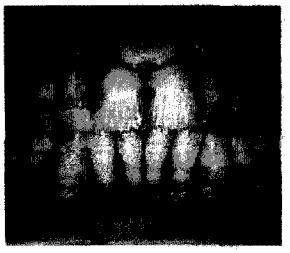
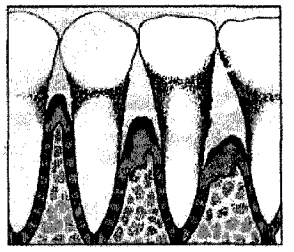




D.





these bacteria, whether it be alone or in combination, have the potential to be periodontopathic (Wang and Ohura, 2002). That is to say that they can initiate periodontal disease once a critical concentration has been reached within the oral cavity. An overgrowth of these periodontal pathogens can result from either a deficiency in the host immune system, or from a modification of the subgingival environment that favours the growth of these pathogens (Marsh, 1989).

2.6 Epidemiology of periodontal and cardiovascular diseases

An epidemiological study conducted by Desvarieux *et al* (Desvarieux et al., 2003) examined the relationship between the chronic infections associated with periodontal disease and cardiovascular disease. The Oral Infections and Vascular Disease Epidemiology Study (INVEST) examined 711 subjects in order to study the hypothesis that periodontal infections predispose induviduals to accelerated progression of atherosclerosis and higher incidences of stroke, myocardial infarction and death due to cardiovascular disease. INVEST was designed as a population-based study investigating the relationship between oral infection, atherosclerosis, and stroke in subjects with no history of stroke or myocardial infarction who were randomly selected from the northern Manhattan area. The subjects received a comprehensive periodontal examination, extensive in-person cardiovascular disease risk factor measurements, and a carotid ultrasound scan of both the right and left carotid arteries. Dentate subjects were categorized according to the extent of current and cumulative periodontal disease, measured by pocket depth and clinical attachment loss respectively. This study reported a significant association between the severity of clinical attachment loss and the prevalence of carotid arterial plaques, with plaques occurring in 46% of the subjects with

mild clinical attachment loss compared with plaques in 60% of subjects with severe clinical attachment loss. These findings suggested a relationship between periodontal and cardiovascular diseases within this population using clinical attachment loss as an indirect measure of periodontal status.

2.6.1 Periodontal microbiota and cardiovascular disease

In a follow-up study conducted by Desvarieux *et al* (Desvarieux *et al.*, 2005) particular attention was paid to direct measurements of periodontal health by investigating the relationship between periodontal microbiota and cardiovascular disease. This INVEST examined 657 subjects in order to study the hypothesis that bacteria causally related to periodontitis would be related to increasing carotid intima-media thickness (IMT). It was designed to assess microbes obtained from the subgingival environment that are known to be etiologically related to periodontitis and a selection of bacteria whose etiologic significance is neutral, using the same type of population-based cohort study that was used in the previous INVEST study. Subjects underwent a complete examination of the oral cavity upon which periodontal health was determined, extensive in-person cardiovascular disease risk factor measurements were completed, and a high resolution ultrasound of the carotid arteries was done to assess carotid IMT. In order to eliminate bias towards diseased sites, plaque samples were taken from the 2 most posterior teeth in each quadrant and quantitatively assessed for 11 known periodontal bacteria by DNA-DNA checkerboard hybridization. The 11 known periodontal bacteria were categorized into 3 groups defined as etiologic (*P gingivalis, T forsythensis, T denticola* and *A actinomycetemcomitans*), putative (*C rectus, E corrodens, F nucleatum, M micros* and *P intermedia*), and health associated (*A naeslundii* and *V parvula*), using

the consensus of the 1996 World Workshop in Periodontics. Patients with a dominance of oral pathogens etiologically related to periodontal disease had thicker carotid IMT than patients with a dominance of oral pathogens from the other two groups, providing direct evidence of a possible role of etiologic periodontal bacteria in atherosclerosis. These results suggest that a possible route to the circulatory system for periodontal bacteria exists, however they provide minimal information as to possible disease mechanisms.

2.6.2 Biological basis for disease association

Dorn *et al* (Dorn *et al.*, 1999) designed a study to determine the biological basis for the association between periodontal bacteria and cardiovascular disease, and to outline a possible mechanism for the disease. The hypothesis that invasion of the coronary artery cells by oral pathogens may start and/or exacerbate the inflammatory response in atherosclerosis was studied using a series of *in vitro* experiments. Dorn *et al* (Dorn *et al.*, 1999) tested the ability of three characterized periodontal pathogens to invade human coronary artery endothelial cells and coronary artery smooth muscle cells. The study found that differences exist in the abilities of the species and strains to invade host cells, indicating that invasion depends on specific bacterium-cell interactions and does not occur with all oral bacteria. Of the three oral pathogens tested in this study, *Porphyromonas gingivalis* was observed to be the most invasive in the presence of both human coronary artery endothelial cells and coronary artery smooth muscle cells. In a follow-up study done by Dorn *et al* (Dorn *et al.*, 2000), 26 different strains of *P gingivalis* were tested for their ability to invade human umbilical vein endothelial cells and KB cells, a human oral epidermoid cell line. All but one of the 26 strains tested were shown to successfully invade human cells, and of the 26 strains tested, *P gingivalis* strain

381 was ranked as the most invasive. This is an important finding as it is *P gingivalis* strain 381 that will be used in our experimental model. The ability of oral bacteria to invade human cell lines, along with the epidemiological relationship between periodontal and cardiovascular diseases, provides significant insight into possible disease mechanisms.

2.6.3 Localization of bacteria at disease sites

Haraszthy *et al* (Haraszthy *et al.*, 2000) studied the relationship between chronic oral infections and cardiovascular disease by employing molecular biological techniques to examine oral pathogens localized at the sites of lesion development. Samples were isolated from 50 human atheroma specimens obtained during carotid endarterectomy to study the hypothesis that chronic oral infections such as those associated with periodontal disease may be involved in the development and progression of atherosclerosis. Atheromas obtained from the subjects were analyzed for species-specific nucleic acids from four bacterial species of periodontal pathogens (*A actinomycetemcomitans, B fordythus, P gingivalis,* and *P intermedia*) by PCR. Analysis showed that 44% of the atherosclerotic plaques generated positive results for at least one of the target periodontal pathogens, with 26% of the samples showing a positive result for the presence of *P gingivalis*. These observations provide evidence that periodontal pathogens, in particular *P gingivalis*, can gain access to, and localize at, sites of lesion development. These results bring into light the possibility that oral infections such as periodontal disease can move into systemic circulation causing a chronic inflammatory insult to the vasculature thereby contributing to the initiation and progression of atherosclerotic lesions.

2.7 *Porphyromonas gingivalis*

Porphyromonas gingivalis (P gingivalis) is an oral black pigmented Gram-negative bacterium that is found in deep periodontal pockets, especially at sites of active periodontal disease (Bodet *et al.*, 2006). It is a major pathogen in the initiation and progression of human periodontal disease, and has been recognized and characterized as a key pathogen and risk factor for periodontal disease. In individuals with severe periodontal disease, approximately 5% of the oral microbiota has been reported to consist of *P gingivalis* (Duncan *et al.*, 2002).

P gingivalis possesses a number of bioactive molecules on its cell surface that contribute to its pathogenesis, such as cytoplasmic membranes, peptidoglycans, outer membrane proteins, lipopolysaccharides (LPS), capsules, and fimbriae which can induce excessive cytokine production (Wang and Ohura, 2002). *P gingivalis* LPS is an important pathogenic component in the initiation and development of periodontal disease (Wang and Ohura, 2002). Degradation of the periodontal tissues in periodontal disease results from inflammatory reactions primarily derived from interactions between the hosts immune system and subgingival bacterial colonies.

Toll-like receptors (TLRs) are pattern-recognition receptors (PRRs) of the innate immune system that recognize specific pathogen associated molecular patterns (PAMPs) through leucine rich repeats (LRRs) at the extracellular level, and therefore represent a key component in the recognition of foreign pathogens (Trinchieri and Sher, 2007). Mammalian TLRs are a family of at least 12 membrane proteins that were originally identified in vertebrates based on their homology to Toll, a molecule that stimulates the

production of antimicrobial proteins in *Drosophila melanogaster* (Trinchieri and Sher, 2007).

Toll-like receptor 4 (TLR4) has been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in most Gram-negative bacteria (Akira and Takeda, 2004). However, there is an increasing amount of evidence that although *P gingivalis* is a Gram-negative bacterium, that it is more closely related to Gram-positive bacteria, and that both TLR2 and TLR4 can induce signal transduction following recognition of different components of the *P gingivalis* bacterium (Akira and Takeda, 2004;Trinchieri and Sher, 2007). Regardless of whether signalling in response to *P gingivalis* occurs through TLR2 or TLR4, upon recognition of specific PAMPs, the TLRs recruit adaptor proteins within the cytoplasm of the cell, such as MyD88, Tirap, Trif and Tram, in order to propagate a signal. These adaptors then activate other proteins within the cell, such as protein kinases (IRAK1, IRAK4, TBK1, IKKi) that lead to an amplification of the signal, which either leads to the induction or suppression of genes involved in the inflammatory response (Akira and Takeda, 2004).

As discussed previously, *P gingivalis* has also been associated with atherosclerosis. Colonization of the subgingival region is due to the ability of the bacterium to adhere to any available substrate, such as extracellular matrix proteins, epithelial cells, and bacteria that have already established themselves as a biofilm on the tooth surface. Secreted or cell-bound enzymes, toxins, and hemolysins play a significant role in the ability of the organism to spread throughout the host and in tissue destruction as it attacks host extracellular matrix proteins, cell adhesion molecules, and protein defences secreted by the immune system, such as cytokines (Bodet *et al.*, 2006). With

evidence demonstrating that *P gingivalis* can invade human oral mucosal cells (Andrian *et al.*, 2004), as well as aortic endothelial cells (Deshpande *et al.*, 1998) *in vitro*, and that *P gingivalis* has been observed within gingival tissues (Rautemaa *et al.*, 2004) *in vivo*, the current view is that the well-vascularized periodontal connective tissues may facilitate the entrance of *P gingivalis* into the blood stream (Bodet *et al.*, 2006). Once inside the host bloodstream, it is possible that *P gingivalis* is able to gain access to sites of atherosclerotic lesion development, potentially contributing to the progression of the disease.

2.8 *P gingivalis* infection and atherosclerosis

2.8.1 Systemic infection

Using an *in vivo* mouse model, Li *et al* (Li et al., 2002) tested the hypothesis that a long-term systemic circulatory challenge with *P gingivalis* can promote and accelerate the development of atherosclerotic lesions. Mice heterozygous for the apolipoprotein E-deficiency ($apoe^{+/-}$) were repeatedly challenged with systemic inoculations of the live periodontal pathogen through intravenous inoculations. (The use of the *apoe* mouse as a model for atherosclerosis is discussed in more detail in Chapter 2.3: "Mouse models for atherosclerosis" on page 6). Mice receiving the challenge of *P gingivalis* showed a statistically significant increase in mean aortic lesion area compared with vehicle controls, providing direct evidence that a long-term systemic challenge with the oral pathogen *P gingivalis* can accelerate atherosclerotic plaque progression. Although this study clearly demonstrated a link between infection by an oral bacteria and atherosclerotic lesion development, the systemic intravenous route of inoculation does not accurately reflect the natural cycle of infection of *P gingivalis*, and therefore as stated

by Li *at al* (Li *et al.*, 2002), it does not, in fact, reproduce infections that occur in patients with periodontal disease.

2.8.2 Oral infection

In order to mimic the oral infection of *P gingivalis* seen in individuals with periodontal disease, Lalla *et al* (Lalla *et al.*, 2003) designed a study to assess the impact of an oral *P gingivalis* infection on the development of atherosclerotic lesions in *apoe*$^{-/-}$ mice. Rather than the intravenous route used by Li *et al* (Li *et al.*, 2002), this group chose to inoculate male *apoe*$^{-/-}$ mice via an oral challenge of *P gingivalis*. Mice receiving the oral challenge demonstrated evidence of a local periodontal infection as measured by an increase in alveolar bone loss, one of the characteristic traits of periodontal disease. Infected mice also displayed evidence of generalized activation of the immune system as measured by an increase in serum immunoglobulin G (IgG) levels. Additionally, PCR analysis of the aortic tissue of infected mice found that *P gingivalis* localized at the site of lesion development within the aorta. In this model infection with live *P gingivalis* intensified the early stages of atherosclerosis development, with analysis revealing a 40% increase in mean lesion area in mice that received the oral challenges compared to control animals. These results demonstrate that oral infection with this periodontal pathogen accelerates early atherosclerotic lesion development, via a route of inoculation that accurately reflects the natural cycle of *P gingivalis* infection in individuals with periodontal disease. The findings reported in this study are significant in that they represent the first experimental evidence that entry of *P gingivalis* through the oral cavity into the hosts system may magnify vascular inflammation and early atherosclerotic lesion formation. With a relationship established between *P gingivalis* and atherosclerosis, the

focus of research is now to identify the inflammatory genes and pathways involved in the progression from periodontal disease to atherosclerosis.

2.8.3 Inflammatory response to *P gingivalis* infection

To investigate the inflammatory response to *P gingivalis*, Bodet et al (Bodet et al., 2006) made use of a human *ex vivo* whole blood model. Whole blood from six healthy individuals was challenged with three different strains of the *P gingivalis* bacterium. Following this challenge, enzyme-linked immunosorbant assay (ELISA) was employed to assess cytokine production. All of the *P gingivalis* strains tested induced a significant increase in several pro-inflammatory cytokine levels, including interleukin (IL)-1β (The significance of IL-1β is discussed in further detail in Section 2.9.1: "The caspase-1 enzyme" on page 24).

While these studies clearly demonstrate that a relationship exists between periodontal disease, in particular *P gingivalis*, and atherosclerosis, they provide little or no evidence as to the mechanism involved in the progression from an oral infection to atherosclerosis.

2.9 Caspases

Caspases are a family of cysteine proteases that carry out critical roles in mammalian apoptosis (Nicholson, 1999;Shi, 2002), and share a stringent specificity for cleaving target proteins at the peptide bond C-terminal to aspartic acid residues (Boatright *et al.*, 2003). Caspases were first implicated in apoptosis through studies conducted using the nematode *Caenorhabditis elegans*. In early studies with cell-death-defective (CED) *C elegans* mutants, it was determined that the *ced-3* gene product was

essential for all developmental apoptotic events in the worm (Creagh *et al.*, 2003). Further studies determined that CED-3 exhibited close sequence homology with the human protease, caspase-1 (also known as ICE, Interleukin-β converting enzyme) (Creagh *et al.*, 2003;Yuan *et al.*, 1993).

Originally, the concerted action of caspases was thought to only be responsible for apoptosis; however, Kuida *et al* (Kuida *et al.*, 1995) reported no defect in apoptosis in mice lacking the caspase-1 enzyme, with the animals proceeding normally through development. Recently a subgroup of the caspase family, termed the inflammatory caspases, have been shown to be involved in inflammation, where they act as pro-cytokine activators and are intricately associated with immune responses to microbial pathogens (Boatright *et al.*, 2003;Martinon and Tschopp, 2004). In humans, caspase-1, -4, and -5, and in mouse caspase-1, -11, and -12 belong to this group of inflammatory caspases which undergo activation during inflammatory responses, with caspase-1 being the best characterized of these caspases (Martinon and Tschopp, 2004).

Caspases are expressed as single chain proenzymes that possess three distinct domains: an N-terminal prodomain, a large subunit, and a small subunit. They are cysteine proteases that exhibit specificity for aspartic acid (Asp) residues (Creagh *et al.*, 2003). Caspases are expressed as inactive proenzymes that are activated following proteolysis at internal aspartic acid residues (Creagh *et al.*, 2003). Given the specificity for caspases to cleave their substrates after motifs that contain Asp residues, it has been suggested that caspases become activated through auto-proteolysis (Creagh *et al.*, 2003).

2.9.1 The caspase-1 enzyme

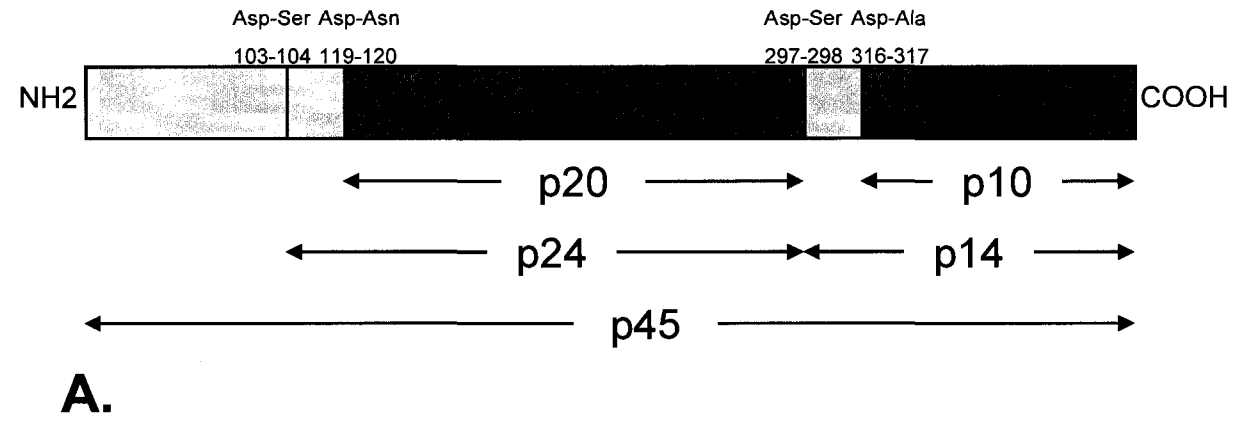
A study conducted by Kuida *et al* (Kuida *et al.*, 1995) identified the enzyme caspase-1 as being responsible for processing the inactive IL-1β precursor into the mature pro-inflammatory cytokine. Using mice deficient in the caspase-1 gene, Kuida *et al* (Kuida *et al.*, 1995) observed that monocytes deficient in this enzyme were not able to produce and secrete mature IL-1β. Studies conducted by Martinon *et al* (Martinon and Tschopp, 2004), Gu *et al* (Gu *et al.*, 1997) and Loppnow *et al* (Loppnow *et al.*, 1998) further investigated the role of caspase-1 in the maturation of other pro-inflammatory cytokines, and reported that in addition to the processing of IL-1β, caspase-1 is also responsible for the activation of IL-18 (IGIF, interferon-γ inducing factor).

2.9.2 Activation of caspase-1

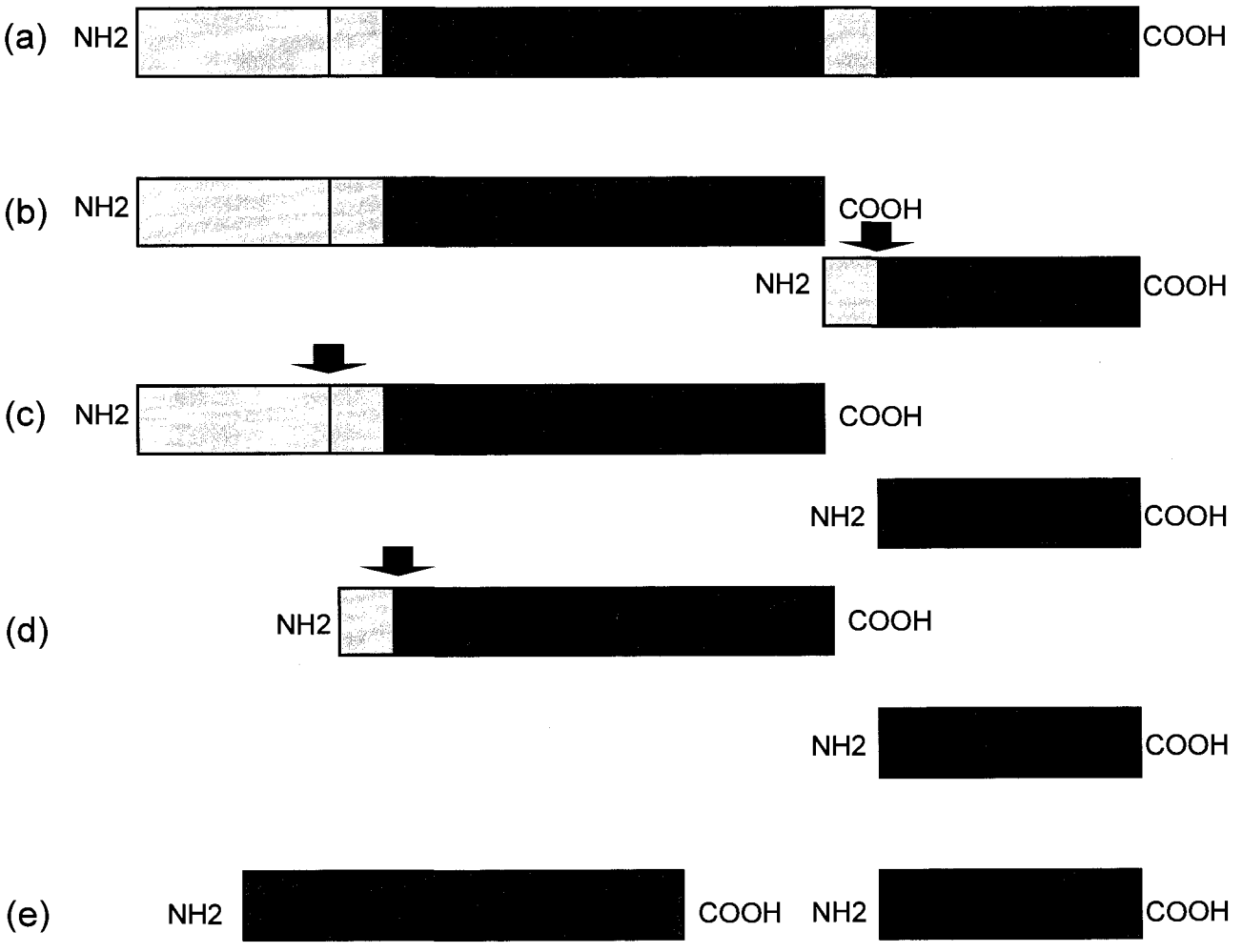
Caspase-1 is a heterodimeric cysteine protease that is constitutively expressed in various cells, including monocyte-derived macrophages, as an inactive 45-kDa precursor (Ramage *et al.*, 1995) (**Figure 3A**). The heterodimeric form consists of a 10-kDa (p10) and 20-kDa (p20) subunit and is generated from the proteolytic cleavage of four peptide bonds within the 45-kDa (p45) precursor (Ramage *et al.*, 1995). The active form of the enzyme exists as a tetramer comprised of two of the p10/p20 heterodimers (Ramage *et al.*, 1995). Ramage *et al* (Ramage *et al.*, 1995) demonstrated that conversion of the p45 proenzyme to the active form of the enzyme occurs in a time-dependent manner through a series of intermediates (**Figure 3B**). The first in the series of cleavage events results in the formation of a p36 and a p14 subunit (Ramage *et al.*, 1995). The next cleavage step occurs in the newly released p14 subunit, which leads to the generation of the p10 subunit

Figure 3: Autocatalytic processing of caspase-1.

(A) The primary structure of human caspase-1. Full length caspase-1 is cleaved at four internal Asp sites to produce the active heterodimeric p10/p20 enzyme, through the p14 and p24 intermediates. **(B)** The autocatalytic processing of the caspase-1 pro-enzyme occurs through a series of intermediates. Sequential cleavage positions are depicted by an arrow, while the shaded regions represent regions removed in the autocatalytic cleavage process. a, intact caspase-1; b, cleavage resulting in the formation of p36 and p14; c, cleavage within p14 to generate the p10; d, cleavage within the p36 releasing the p24; e, cleavage within the p24 to generate the p20. (Figure adapted from Ramage *et al* (Ramage *et al.*, 1995)).


Asp-Ser Asp-Asn
103-104 119-120
Asp-Ser Asp-Ala
297-298 316-317
NH2
COOH
p20
p10
p24
p14
p45

A.


(a)
NH2
COOH
(b)
NH2
COOH
NH2
COOH
(c)
NH2
COOH
NH2
COOH
(d)
NH2
COOH
NH2
COOH
(e)
NH2
COOH
NH2
COOH

B.

(Ramage *et al.*, 1995). Cleavage within the p36 subunit generates a p24 intermediate which is then further cleaved to generate the p20 subunit (Ramage *et al.*, 1995).

2.9.3 Regulation of caspase-1 activation

To date, a detailed mechanism of the steps involved in the activation of the pro-inflammatory enzyme caspase-1 has not been well defined. Recently, a molecular platform termed the 'inflammasome' was identified and characterized as an intracellular caspase activating scaffold. Inflammasomes are 700 kDa, multiprotein complexes which recruit inflammatory caspases, thereby triggering their activation (Petrilli *et al.*, 2005). The inflammasomes are defined by the distinguishing nucleotide-binding and oligomerization domain-like receptor (NLR) family member that links the recognition of certain pathogens to the autoactivation of caspase-1 (Lamkanfi *et al.*, 2007). The inflammasome contains three distinct structural domains: (a) a region that interacts with the pro-domain of the caspase (termed the adaptor protein), (b) an oligomerization motif that facilitates the formation of dimers, and (c) a domain that regulates its activity (Martinon and Tschopp, 2004).

In response to specific bacterial or microbial components, the NLR proteins assemble a caspase-1 activating inflammasome complex (Lamkanfi *et al.*, 2007) (**Figure 4**). The caspase activation and recruitment domain (CARD) in the N-terminal region of the NLR protein interacts with the CARD of an adaptor protein, ASC (apoptosis-associated speck-like protein containing a CARD), which in turn interacts with the CARD in the pro-domain of caspase-1 (Lamkanfi *et al.*, 2007;Mariathasan *et al.*, 2004). ASC is therefore an essential component in the formation and function of the inflammasome complexes. The inflammasome complex that uses ASC as its adaptor

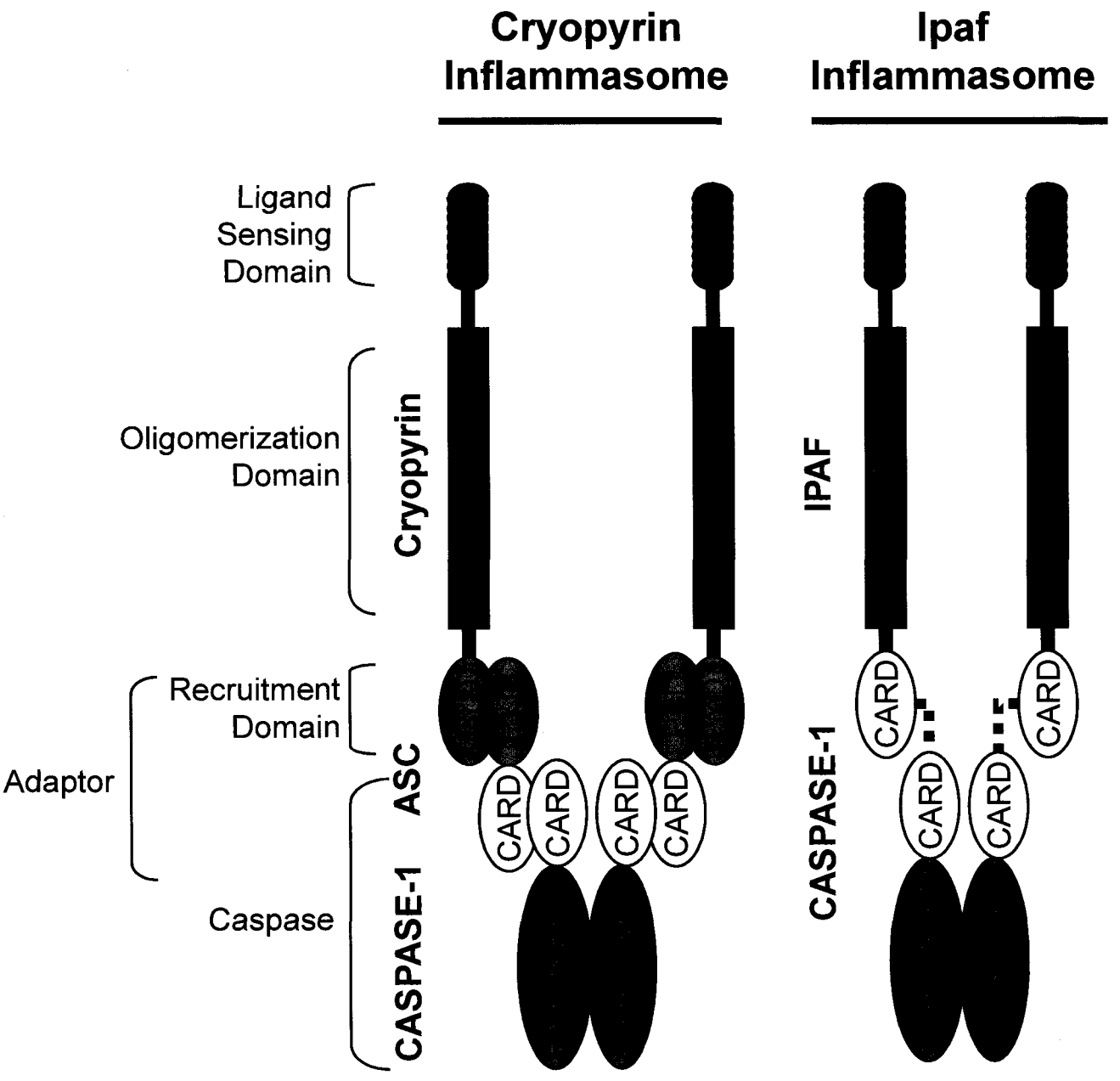
protein is known as the Cryopyrin inflammasome. Another inflammasome adaptor protein that has been implicated in the interaction with caspase-1 and has also been proposed to modulate its activity is Ipaf (ICE-protease-activating factor) (Lamkanfi *et al.*, 2007;Mariathasan *et al.*, 2004) (**Figure 4**). Ipaf has a CARD in its amino-terminal domain that has been suggested to associate with either the CARD of caspase-1 or directly with the adaptor protein ASC itself (Poyet *et al.*, 2001). The inflammasome complex containing Ipaf is simply termed the Ipaf inflammasome. The NLR family members also contain a nucleotide binding oligomerization domain (NOD) that facilitates the formation of caspase-1 dimers (Martinon and Tschopp, 2004).

Inflammasomes exist in the cytoplasm as inactive monomers which are activated only upon receipt of a specific signal through the binding of a ligand (Martinon and Tschopp, 2004). Little is known about the natural stimuli that lead to the assembly and subsequent activation of the inflammasome (Petrilli et al., 2005). It is thought that activation of the inflammasome occurs through the recognition of pathogen-associated molecular patterns (PAMPs) by leucine rich repeats (LRRs) located in the C-terminal region of the NLR protein (Petrilli et al., 2005).

Since the inflammasome functions at the intracellular level upon recognition of specific bacterial components, it is likely that there is an extracellular component that sends an intracellular signal to the inflammasome to trigger its activation. As discussed previously, Toll-like receptors (TLRs) are pattern-recognition receptors (PRRs) of the innate immune system that recognize specific PAMPs through LRRs at the extracellular level, and therefore represent a key component in the recognition of foreign pathogens (Trinchieri and Sher, 2007). It is therefore plausible that TLRs are one of the

Figure 4: Schematic model of proposed inflammasomes activating caspase-1.

The ligand sensing region initiates the formation of oligomers by the oligomerization domain (NOD) of the NLR proteins Cryopyrin and Ipaf (in the Crypoyrin and Ipaf inflammasomes respectively), while PYD-PYD and CARD-CARD interactions recruit either the adaptor protein ASC (in the Cryopyrin inflammasome) or the inflammatory caspase-1 (in the Ipaf inflammasome). The brackets identify the various components of the inflammasome: the ligand sensing domain, the oligomerization domain, the adaptor, and the caspase. (Reprinted with permission from the Journal of Leukocyte Biology, Lamkanfi *et al* (Lamkanfi *et al.*, 2007), copyright 2007).


Cryopyrin Inflammasome
Ipaf Inflammasome
Ligand Sensing Domain
Oligomerization Domain
Cryopyrin
Recruitment Domain
Adaptor
ASC
Caspase
CASPASE-1
IPAF
CARD
CARD
CARD
CARD
CARD
CARD
CARD
CARD
CASPASE-1

extracellular signals required for the formation of the intracellular inflammasome upon recognition of *P gingivalis* and the subsequent activation of caspase-1.

2.10 Preliminary studies

Caspase-1 was originally identified as the enzyme responsible for the maturation of the pro-inflammatory cytokines IL-1β (Kuida *et al.*, 1995) and IL-18 (Gu *et al.*, 1997;Loppnow *et al.*, 1998;Martinon and Tschopp, 2004). As outlined below, recently a number of laboratories have demonstrated that both IL-1β and IL-18 promote atherosclerosis in the apolipoprotein E deficient (*apoe*$^{-/-}$) mouse model.

Kirii *et al* (Kirii *et al.*, 2003) designed a study to examine the role of IL-1β in the development of atherosclerotic lesions using an *apoe*$^{-/-}$ mouse model deficient in endogenous IL-1β. IL-1β is a pro-inflammatory cytokine that is responsible for a variety of actions, including initiation of cyclooxygenase type 2 (Lee *et al.*, 2004), type 2 phospholipase A (Pascual *et al.*, 2003), and inducible nitric oxide synthase (Hashimoto *et al.*, 2003). In their study, it was found that there was a decrease in atherosclerotic lesion formation of approximately 30% in mice lacking IL-1β, as compared to those competent for IL-1β. These results demonstrate that a lack of IL-1β decreases the severity of atherosclerosis in *apoe*$^{-/-}$ mice.

Given that caspase-1 is also responsible for the activation of another pro-inflammatory cytokine, IL-18, a preliminary study conducted by our laboratory was designed to examine the role of IL-18 in the development of atherosclerosis using a mouse model that was deficient in IL-18. IL-18 plays a key role in innate immune responses through the activation of macrophages (Munder *et al.*, 1998), T-helper type 1

cells (Cooper *et al.*, 1997;Decken *et al.*, 1998), and natural killer cells (Kawakami *et al.*, 2000). It was found that mice lacking endogenous IL-18 demonstrated approximately a 40% reduction in mean atherosclerotic lesion size as compared to control mice (Whitman *et al.*, 2002a). These preliminary results suggest a role for IL-18 in the development of atherosclerosis.

Combining the outcome of these two studies, the results are encouraging as they outline a possible mechanism for *P gingivalis* mediating atherosclerotic lesion development through the activation of caspase-1 and subsequent maturation of IL-18 and IL-1β.

2.10.1 Caspase-1 deficiency decreases atherosclerosis in a mouse model

Previous studies have shown that IL-18 promotes atherosclerosis in an *apoe*$^{-/-}$ mouse model of atherosclerosis (Whitman *et al.*, 2002a), and that a deficiency in IL-1β prevents the development of atherosclerotic lesions in *apoe*$^{-/-}$ mice (Kirii *et al.*, 2003). Since the caspase-1 enzyme is primarily responsible for the maturation of both IL-18 and IL-1β, our laboratory conducted a preliminary study to investigate the role of caspase-1 in the development of atherosclerosis.

In the Whitman lab, it was suspected that endogenous activity of the caspase-1 enzyme would promote atherosclerosis, given that it is a potent stimulator of the pro-inflammatory cytokines IL-18 and IL-1β *in vivo*. In a preliminary study, male and female *apoe*$^{-/-}$ mice that were either caspase-1$^{+/+}$ or caspase-1$^{-/-}$ were fed a normal rodent diet for 26 weeks from the time of weaning, or a diet supplemented with 1.5% (w/w) cholesterol and 16% (w/w) butterfat for 8 weeks once the mice had reached 8 weeks of age. Feeding

the mice a normal diet for 26 weeks has been previously shown to cause the formation of early atherosclerotic lesions that consist mainly of macrophage-derived foam cells, while feeding the mice a high-fat, cholesterol enriched diet for 8 weeks has been previously shown to cause the formation of atherosclerotic lesions that are in both the early and advanced stages of development, containing well defined necrotic cores with or without an overlying fibrous cap. By using two different dietary protocols in this preliminary study we were able to look at the effect that endogenous caspase-1 deficiency has on the development of both early and advanced stage atherosclerotic lesions (unpublished data).

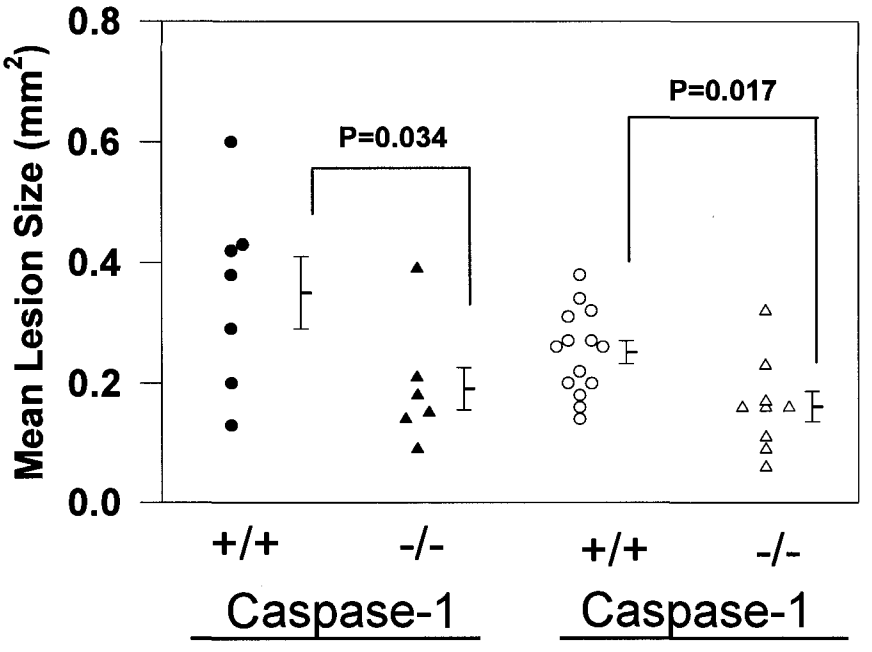
By employing an *apoe*$^{-/-}$ mouse model deficient in the endogenous caspase-1 enzyme, we observed that loss of endogenous caspase-1 function significantly decreased lesion size by approximately 35-45% in the ascending aorta of both male and female mice fed either the low or high fat diets (**Figure 5A & B**). Based on the lesion staining patterns for neutral lipid (Sudan IV), collagen (Gomori Trichrome), and macrophages (immunocytochemistry), we determined that the majority of lesions from both male and female caspase-1$^{+/+}$ and caspase-1$^{-/-}$ mice feed the low-fat diet were at the early stage of lesion development as these lesions consisted mostly of macrophage-derived foam cells. Necrotic cores with or without an overlying fibrous cap were present in some lesions from caspase-1$^{+/+}$ mice fed the high fat diet, while none of these advanced stage lesions could be found in caspase-1$^{-/-}$ mice fed the same diet.

By examining the effect of a caspase-1 deficiency in both genders of *apoe*$^{-/-}$ mice, we were able to show that functionally active endogenous caspase-1 contributes to the development of atherosclerotic lesions. Furthermore, we were able to show that a lack of endogenous caspase-1 activity slowed the development of both early and advanced

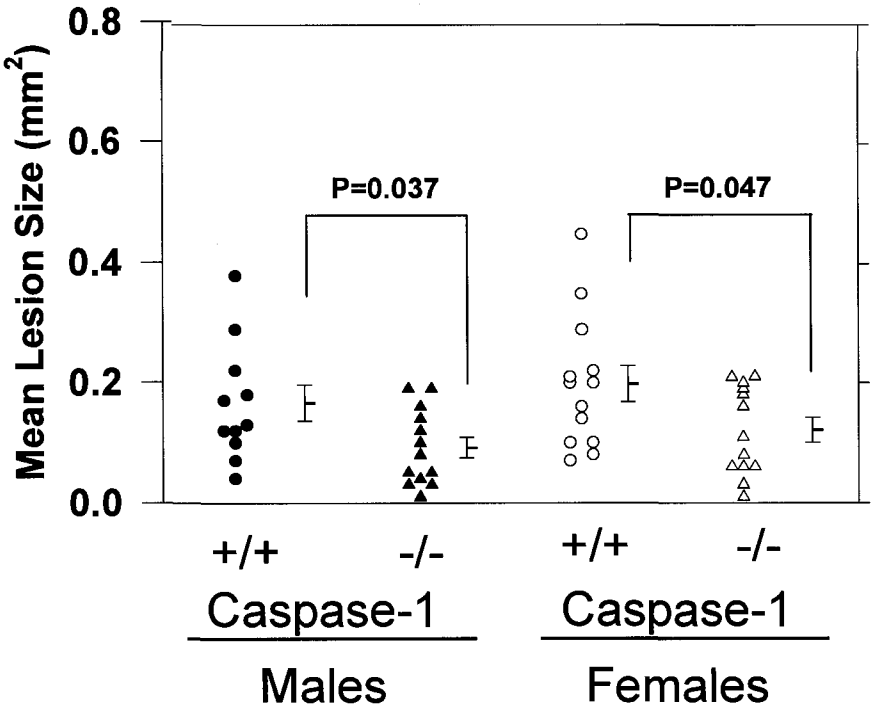
atherosclerotic stage lesions in both male and female atherosclerosis susceptible *apoe*$^{-/-}$ mice. These preliminary results are essential in outlining a role for caspase-1 in the development of atherosclerotic lesions.

Figure 5: Caspase-1 deficiency decreases atherosclerosis *in vivo*.

The extent of atherosclerotic lesion development in the ascending aorta of male and female *apoe*$^{-/-}$ mice that were either caspase-1$^{+/+}$ or caspase-1$^{-/-}$ was determined (using the same method as described in Chapter 4 : "Methods" in section 4.11: "Tissue cryosectioning and atherosclerotic lesion analysis" on page 46). (**A**) Atherosclerotic lesion size quantified after the mice had been fed a high-fat diet for 8 weeks. (**B**) Atherosclerotic lesion size quantified after the mice had been fed a low-fat diet for 26 weeks. Values of individual mice are represented as circles (caspase-1$^{+/+}$) and triangles (caspase-1$^{-/-}$) for both males (solid symbols) and females (open symbols). The mean lesion size of each group of mice is presented as a single horizontal line to the right of each grouping of symbols, with error bars denoting SEM (unpublished data).


Mean Lesion Size (mm²)
0.8
0.6
0.4
0.2
0.0
P=0.034
P=0.017
+/+
-/-
+/+
-/-
Caspase-1
Caspase-1
Males
Females

A.


Mean Lesion Size (mm²)
0.8
0.6
0.4
0.2
0.0
P=0.037
P=0.047
+/+
-/-
+/+
-/-
Caspase-1
Caspase-1
Males
Females

B.

CHAPTER 3 : RATIONALE AND OBJECTIVES

3.1 Rationale

The rationale for the present study is outlined in **Figure 6** on page 36. Several epidemiological studies have drawn a link between periodontal disease and atherosclerosis (Desvarieux *et al.*, 2003;Desvarieux *et al.*, 2005). *P gingivalis* is a key pathogen in periodontal disease that has been identified in atherosclerotic lesions of both humans (Haraszthy *et al.*, 2000) and mice (Lalla *et al.*, 2003). Previous studies have demonstrated that an oral infection with *P gingivalis* is capable of increasing atherosclerotic lesion development in a mouse model for atherosclerosis (Lalla *et al.*, 2003). *P gingivalis* has been shown to increase the expression of the pro-inflammatory cytokines IL-18 and IL-1β (Bodet *et al.*, 2006), both of which have been shown by our lab and by others to promote atherosclerosis (Kirii *et al.*, 2003). The enzyme responsible for the maturation of both of these cytokines into their mature forms has been identified as caspase-1 (Gu *et al.*, 1997;Kuida *et al.*, 1995;Loppnow *et al.*, 1998;Martinon and Tschopp, 2004). Preliminary studies conducted by the Whitman lab have demonstrated the ability of caspase-1 to promote atherosclerosis in a mouse model for the disease. What remains unknown is whether the ability of *P gingivalis* to promote atherosclerotic lesion development is linked to its ability to bring about caspase-1 activation.

3.2 Objective and hypothesis

Objective #1. To determine the cytokines produced by macrophages exposed to live *P gingivalis.*

Hypothesis #1. Infection of peritoneal macrophages isolated from caspase-1 competent mice with *P gingivalis* will lead to increased expression of inflammatory cytokines known to be associated with atherosclerosis.

Objective #2. To determine whether the periodontal pathogen, *P gingivalis*, is capable of activating the caspase-1 enzyme

Hypothesis #2. Infection of peritoneal macrophages isolated from caspase-1 competent mice with *P gingivalis* will lead to activation of the caspase-1 enzyme.

Objective #3. To determine whether the periodontal pathogen, *P gingivalis,* is capable of localizing at sites of lesion development.

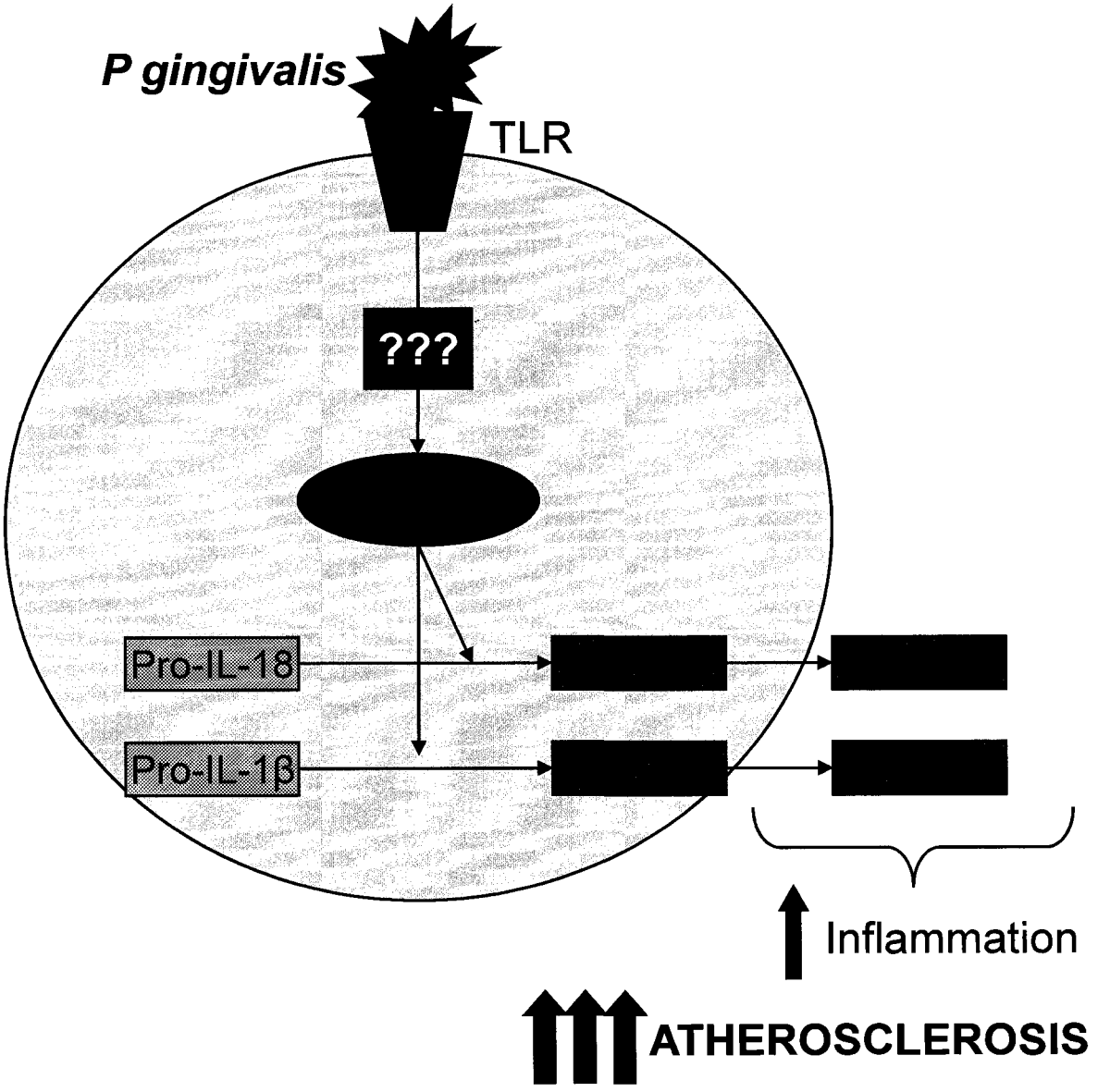
Hypothesis #3. Mice orally challenged with *P gingivalis* will display evidence of *P gingivalis* localization at sites of lesion development upon examination by electron microscopy.

Objective #4. To determine whether the periodontal pathogen, *P gingivalis*, is able to promote atherosclerosis through the activation of caspase-1.

Hypothesis #4. Caspase-1 deficient mice will be less susceptible to the development of atherosclerotic lesions following an oral infection with *P gingivalis* than their caspase-1 competent littermates.

Figure 6: Caspase-1 and *P gingivalis* mediated atherosclerosis - Rationale.

P gingivalis is a key pathogen in periodontal disease that has been identified within human and mouse atherosclerotic lesions. Oral infection with *P gingivalis* increases lesion development in a mouse model for the disease. *P gingivalis* increases the expression of IL-18 and IL-1β *in vitro*, both of which promote atherosclerosis *in vivo*. Caspase-1 is the enzyme responsible for the maturation of IL-18 and IL-1β. Preliminary studies identify a role for caspase-1 in lesion development. What remains to be elucidated is whether the ability of *P gingivalis* to promote atherosclerosis is linked to its ability to bring about caspase-1 activation (denoted by the black box).


P gingivalis
TLR
???
Pro-IL-18
Pro-IL-1β
Inflammation
ATHEROSCLEROSIS

CHAPTER 4 : METHODS

4.1 Bacterial strains and culture conditions

Porphyromonas gingivalis strain 381 (ATCC, cat # 33277) was grown in Difco Anaerobic Broth (Fisher Scientific, Ottawa, ON) under an anaerobic atmosphere (85% N_2, 10% H_2, 5% CO_2) at 37°C. The presence of *P gingivalis* was confirmed by PCR using a pair of primers corresponding to *P gingivalis* specific sequences on 16S ribosomal RNA at the base position 729-1132. The 2 primers used to amplify the 404-base pair region of the 16S ribosomal RNA of *P gingivalis* were 5'-AGG CAG CTT GCC ATA CTG CG-3' and 5'-ACT GTT AGC AAC TAC CGA TGT-3'. Briefly, bacterial samples were pelleted by centrifugation at 4000 rpm for 4 minutes. The supernatant was removed from the sample, the pellet was incubated with Direct PCR Lysis Reagent (Viagen Biotechnology, Los Angeles, CA), and incubated overnight at 55°C to complete the DNA extraction process. PCR was performed for 36 cycles at an annealing temperature of 56°C to 60°C and amplified PCR products were detected by gel electrophoresis using a 1.5% agarose gel.

Salmonella typhimurium was used as a control in various experiments throughout this study. *Salmonella typhimurium* sl1344 (Salmonella Genetic Stock Center, University of Calgary, AB) was grown in LB media under atmospheric conditions at 37°C. For infection experiments, bacteria were grown until the cultures reached an optical density of 0.8 at 660 nm and were then pelleted by centrifugation and resuspended in culture media lacking antibiotics.

4.2 Mouse peritoneal macrophage isolation and culture

Mice were injected with 3 mL of sterile 4% thioglycollate medium (Sigma-Aldrich, St. Louis, MO) into the peritoneal cavity. Four days thereafter, thioglycollate-elicited peritoneal macrophages were harvested by peritoneal lavage with 9 mL of culture medium. Macrophages were pooled and centrifuged at a speed of 4000 rpm for 10 minutes at 4°C. Macrophages were resuspended in 10 mL of culture medium and counted using the *ViCell* cell counter. A concentration of 1 x 10^6 cells/mL were plated in each well of a culture plate in medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Grand Island, NY) and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY). After a 2 hour incubation at 37°C in an atmosphere containing 5% CO_2, non-adherent cells were removed by washing with serum-free culture medium. Adherent macrophages were cultured for 3 days before being exposed to stimulants.

4.3 Electron microscopy

Protocols were carried out as described previously (Dorn *et al.*, 2000). For these experiments, mouse peritoneal macrophage isolation and culture were carried out using DMEM culture media and cells were plated in a 10cm culture dish in a total volume of 10mL. For the invasion assay, *P gingivalis* was grown to an optical density of 0.8 at 660 nm which corresponds to a concentration of 10^9 bacterial cells/mL. Bacteria were pelleted by centrifugation at 4000 rpm for 20 mins and resuspended in 1 mL of antibiotic-free DMEM. Mouse peritoneal macrophages were washed 3 times with phosphate-buffered saline (PBS) prior to incubation with the bacterial suspension at 37°C for 90 mins under aerobic conditions (5% CO_2). In order to more closely mimic *in vivo*

conditions, the bacteria were not centrifuged onto the cells to promote intimate contact. The medium was removed from the cells after 90 mins and the cells were washed 2 times with PBS. Cells were then fixed in 1.6% gluteraldehyde in 0.1M Na cacodylate buffer (pH=7.4) and post-fixed in 1% osmium tetroxide in 0.1M Na cacodylate buffer. The sample was then stained in 3% aqueous uranyl acetate, dehydrated in an ascending series of ethanol washes, and subsequently infiltrated and embedded in Spurr epoxy resin. Sections were cut 0.5 μm thick using a Reichert Ultracut E and stained with methylene blue. Ultrathin sections were cut and counterstained with Reynolds lead citrate and examined using a Jeol 1230 transmission electron microscope.

For analysis of mouse hearts using electron microscopy hearts were dissected from one male caspase-$1^{+/+}$ x *apoe*$^{-/-}$ control mouse and one male caspase-$1^{+/+}$ x *apoe*$^{-/-}$ mouse that had been orally challenged with the *P gingivalis* bacterium. Hearts were then fixed in 1.6% gluteraldehyde in 0.1M Na cacodylate buffer (pH=7.4) and post-fixed in 1% osmium tetroxide in 0.1M Na cacodylate buffer. The sample was then stained in 3% aqueous uranyl acetate, dehydrated in an ascending series of ethanol washes, and subsequently infiltrated and embedded in Spurr epoxy resin. Sections were cut 0.5 μm thick using a Reichert Ultracut E and stained with methylene blue. Ultrathin sections were cut and counterstained with Reynolds lead citrate and examined using a Jeol 1230 transmission electron microscope.

4.4 Caspase-1 activation assay

Protocols were carried out as described previously (Mariathasan *et al.*, 2004). For these experiments, mouse peritoneal macrophage isolation and culture were carried out

using Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY) and cells were plated in a 6-well culture plate in a total volume of 2 mL/well. Following macrophage isolation and culture, live *Porphyromonas gingivalis* or *Salmonella typhimurium* were added to culture media that lacked antibiotics, and subsequently added to wells containing macrophages, resulting in a final multiplicity of infection (MOI) of 50:1. Infected macrophages were lysed in RIPA buffer (10mM phosphate buffer pH 7.4, 150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail. Lysates were resolved in 12.5% Tris-glycine gels and transferred to PVDF membranes (BioRad Labs, Hercules, CA). Membranes were blocked with 5% skim milk for 1 hour with rocking at room temperature. Membranes were incubated overnight with a 1/100 dilution of the antibody directed against the p10 subunit of the caspase-1 enzyme (sc-514, Santa Cruz Biotechnology, Inc., Santa Cruz, California), followed by a 2 hour incubation with a 1/5000 dilution of the HRP conjugated antibody (sc-2004, Santa Cruz Biotechnology, Inc., Santa Cruz, California). Membranes were then visualized using the FluroChem HD9900 imager (Alpha Innotech, San Leandro, CA).

4.5 Foam cell assay

For these experiments, mouse peritoneal macrophage isolation and culture were carried out using RPMI 1640 medium and cells were plated on an 8-chambered slide in a total volume of 200 μL/well. Culture media was changed 1 hour before macrophages were exposed to stimulants. Mouse peritoneal macrophages were serum starved for 2 hours in RPMI 1640 medium lacking fetal bovine serum, following which they were incubated with modified human lipoprotein oxidized LDL (oxLDL) (Intracel, Frederick,

MD) at a concentration of 100 μg/mL for 24-hours in an atmosphere containing 5% CO_2. Macrophages were then washed with warm serum free RPMI 1640 medium. Cells were fixed in 4% paraformaldehyde for 30 mins, and stained with filtered Oil Red O for 45 mins at room temperature, and washed 3 times with 1X PBS. Wells were removed from the slide and the slides mounted and coverslipped using Aquatex (EMD Chemicals, Gibbstown, NJ) mounting medium. These experiments were run three times in duplicate. Results from this assay were visualized under the light microscope.

4.6 Cytokine profiling

Protocols were carried out as described previously (Zhou *et al.*, 2005). For these experiments, mouse peritoneal macrophage isolation and culture were carried out using RPMI 1640 medium (Invitrogen, Grand Island, NY) and cells were plated in a 6-well culture plate in a total volume of 2 mL/well. Culture media was changed 1 hour before macrophages were exposed to stimulants. Mouse peritoneal macrophages were serum-starved for 2 hours in RPMI 1640 medium lacking fetal bovine serum, following which they were incubated with oxidized human LDL (oxLDL) (Intracel, Frederick, MD) at a concentration of 100 μg/mL for 24-hours in an atmosphere containing 5% CO_2. Macrophages were then washed with warm serum-free RPMI 1640 medium. Live *Porphyromonas gingivalis* (10^8 to 10^9 bacterial cells) was added to culture media that lacked antibiotics, and subsequently added to wells containing macrophages, resulting in a final multiplicity of infection (MOI) of 50:1. Cell culture media was collected following 24 hours of infection in an atmosphere containing 5% CO_2. These experiments were run three times in duplicate.

Culture media was analyzed using RayBio Mouse Cytokine Antibody Array III (RayBiotech Inc., Norcross, GA) according to manufacturer's instructions. Briefly, array membranes were blocked in 1X blocking buffer for 30 min and then incubated with 1 mL of sample at room temperature for 2 h. Samples were decanted and membranes were washed three times with 2 mL 1X wash buffer I, followed by two washes with 2 mL of 1X wash buffer II at room temperature. Membranes were then incubated with a 1:250 dilution of the biotin-conjugated primary antibody at 4°C overnight. Array membranes were washed as described previously, and then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated streptavidin for 60 min at room temperature. Membranes were once again washed, and then exposed to a peroxidase substrate (detection buffers C and D; RayBiotech Inc.) for 5 min before imaging. Membranes were visualized using the FluorChem HD9900 imager and analyzed with the imager software. Biotin-conjugated immunoglobulin G served as a positive control at six spots on the array membrane, where it was used to both identify membrane orientation and to normalize results from different membranes being compared. For each membrane cytokine expression is reported as an integrated density value (IDV) normalized to the positive control.

4.7 Animals

All animal studies were performed in accordance with the policies and guidelines of the University of Ottawa Animal Care Committee. This study was designed to compare lesion development in sibling male and female *apoe*$^{-/-}$ mice that are caspase-1 $^{+/+}$ versus $^{-/-}$ following infection with *P gingivalis*. Male caspase -1 $^{-/-}$ mice were bred with female atherosclerosis susceptible *apoe*$^{-/-}$ mice originally obtained from the Jackson

Laboratory, (ME, USA), and maintained in the Animal Care Facility at the University of Ottawa Heart Institute. Both strains of mice were backcrossed onto the C57BL/6 background for more than 10 generations. After the original cross, the F1 heterozygotes were mated with apo-E $^{-/-}$ mice to obtain an F2 generation that served as the breeding colony for this experiment. The F3 progeny from crossing caspase-1 $^{+/-}$ X *apoe*$^{-/-}$, were genotyped and male and female caspase-1 $^{-/-}$ X *apoe*$^{-/-}$ and caspase-1 $^{+/+}$ X *apoe*$^{-/-}$ littermates were used for these experiments. PCR was carried out on DNA from mouse tail samples to screen for the apoE and caspase-1 genes, under reaction conditions recommended by the Jackson Laboratory.

4.8 Inoculation of mice with *P. gingivalis*

P gingivalis strain 381 (ATCC cat. #33277) was grown as described previously (Nassar *et al.*, 2002). Inoculation of *apoe*$^{-/-}$ mice will be done essentially as described by Lalla *et al* (Lalla *et al.*, 2003). Briefly, *P gingivalis* was maintained on Anaerobic Agar plates (VWR, Mississauga, ON) incubated at 37°C for 3 to 5 days in an anaerobic chamber kept under the gas ratios: 85% N_2, 5% H_2, and 10% CO_2. For infection of the mice, *P gingivalis* was transferred from plates to Difco Anaerobe Broth (Fisher Scientific, Ottawa, ON) and grown for an additional 24 hours or until the optical density at 660 nm reached 1.0 as described previously (Nassar *et al.*, 2002). At age 5 weeks, mice received 4 daily doses of ampicillin/kanamycin (2 mg per drug per day) administered via an oral topical application, in an attempt to facilitate the subsequent bacterial inoculation and enhance colonization. Six-week old male and female *apoe*$^{-/-}$ mice that were caspase-1 $^{+/+}$ versus $^{-/-}$ were challenged with 10^{10} bacterial cells of *P gingivalis*, administered via oral topical application over a 3-week period for a total of 15

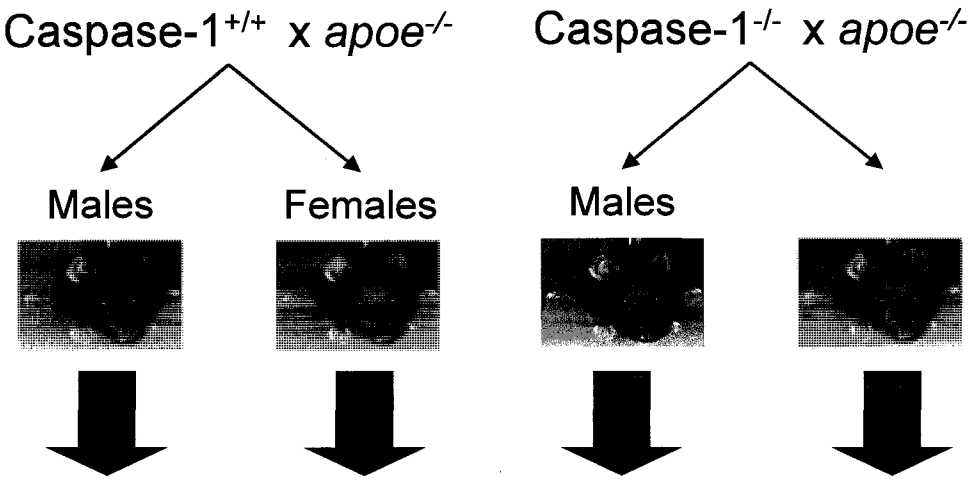
inoculations. Control *apoe*$^{-/-}$ mice that were either caspase-1 $^{+/+}$ or $^{-/-}$ received antibiotics and vehicle on the same schedule. At age 17 weeks, all mice were euthanized and atherosclerotic lesion analysis began. Experimental set-up is outlined in **Figure 7**.

4.9 Plasma cholesterol and lipoprotein profiles

At the end of the dietary period, terminal blood samples were collected from each mouse by a puncture to the right ventricle. A commercially available calorimetric assay (Wako Bioporducts, Richmond, VA) was used to determine serum total cholesterol concentrations present in terminal blood samples of the mice. Serum samples (60 ul) from each mouse were used to determine individual lipoprotein cholesterol distributions

Figure 7: Experimental set-up for *in vivo* mouse study.

Schematic diagram of the experimental set-up for the *in vivo* mouse study. Briefly, 5 week old male and female mice that were either caspase-$1^{+/+}$ x *apoe*$^{-/-}$ or caspase-$1^{-/-}$ x *apoe*$^{-/-}$ received 4 daily doses of ampicillin/kanamycin (2 mg per drug per day) administered via an oral and anal topical application. Six-week old mice were orally challenged with 10^{10} bacterial cells of *P gingivalis* per day, over a 3 week period for a total of 15 inoculations. Control mice received antibiotics on the same schedule. At age 17 weeks, all mice were euthanized, tissues were collected, and atherosclerotic lesion analysis was performed.



5 weeks of age: 4 daily doses of ampicillin/kanamycin (2mg/drug/day)

6 weeks of age: Oral challenge with 10^{10} CFU of *P gingivalis* daily (total of 15 inoculations)

17 weeks of age: Tissue collection

following fractionation of the serum by size exclusion chromatography (HPLC made by Gilson, Middleton, WI) using a Superose 6 column (Pharmacia LKB Biotechnology, Uppsala, Sweden). The fractions were collected and cholesterol concentrations were determined using an enzymatic assay kit for cholesterol assays (Wako Bioporducts, Richmond, VA).

4.10 Tissue collection

Following the dietary period, all mice were perfused with PBS by means of a puncture to the left ventricle, with the perfusate being drained from a severed right atrium. The hearts were separated from the aorta at the base, placed in a plastic base mold, embedded in optimum cutting temperature (OCT) medium, and snap frozen on a metal plate cooled in liquid nitrogen. Samples were wrapped in Parafilm and stored at -20°C.

4.11 Tissue cryosectioning and atherosclerotic lesion analysis

The extent of atherosclerotic lesion development was determined through analysis of the ascending aorta. To determine atherosclerotic size within the ascending aorta, the mean lesion area was derived from 4 serial Sudan IV stained sections that were cut 10 μm thick, and collected 100 μm apart over a 1 mm segment of the aortic root. The mean lesion area was taken as the lesion size for each mouse (Whitman *et al.*, 2000;Whitman *et al.*, 2002b;Whitman *et al.*, 2002a;Whitman *et al.*, 2004), and analysis of the lesions began at the region where the aortic sinus becomes the ascending aorta. Using the Sudan IV stained sections as a guide, the lesion area defined as intimal tissue within the internal elastic lamina was determined from images created by using the digital CoolSNAP *cf*

camera (Roper Scientific Inc., Duluth, GA) using Image-Pro software (Media Cybernetics, Silver Springs, MD).

4.12 Quantification of alveolar bone loss

Evaluation of the extent of *P. gingivalis* induced periodontal destruction was determined by the measurement of alveolar bone loss in the mice, as described previously (Lalla *et al.*, 1998;Lalla *et al.*, 2003) with slight modifications. The mandibles were removed under a dissecting microscope, and using microdissecting forceps and a scalpel with a no. 15 blade, the lingual gingival tissue was removed from each posterior quadrant. Beginning with a horizontal sulcular incision, full thickness flaps were reflected, vertical release incisions were made, and the gingival tissue removed. A horizontal incision just below the mucogingival junction was then used to separate the tissue. Following removal of the gingival tissue, mandibles were hemisected, exposed to 3% H_2O_2 overnight and then mechanically defleshed. The jaws, with the lingual surface of each half mandible exposed, were then embedded in Play-Doh. The buccal and lingual cusps were superimposed during embedding to remove angulation as a variable. The jaws were photographed using the digital CoolSNAP *cf* camera. For each mouse, alveolar bone loss was defined as the total area between the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) for a total of 6 mandibular posterior teeth. Analysis was then performed by a blinded investigator using Image-Pro software.

4.13 Statistical analysis

Data analysis was performed using SigmaStat 2.03 software (SPSS Inc., Chicago, IL). For each parameter, the mean and standard error of mean was calculated. Statistical

analysis between groups was evaluated by an unpaired Student's *t*-test, with a 2-tailed distribution. Power calculations from previous studies indicated that the outlined experiments would use a sufficient number of animals to attain a power of 0.8 for a 20% change in lesion size and provide statistically reliable results if no effects of atherosclerosis are observed. Values where $p \leq 0.05$ were considered statistically significant.

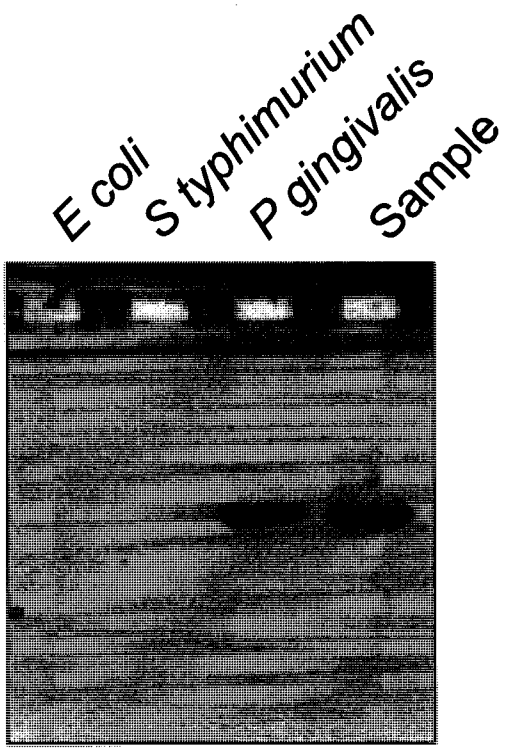
CHAPTER 5 : RESULTS

5.1 Quantification of *P gingivalis*

The current study was designed to examine the role of caspase-1 in *P gingivalis* mediated atherosclerotic lesion development. As such, it was essential to ensure not only that the bacteria with which the mice were receiving an oral challenge were in fact the right bacteria, but also to determine the amount of bacteria with which each mouse was being orally challenged and to ensure that the bacteria was viable. To address the first concern, primers were used that were specific to *P gingivalis* in order detect the presence of the bacteria grown in anaerobic broth, under anaerobic conditions. Samples of the bacteria were taken on a monthly basis throughout the entire experimental period. Following DNA extraction, PCR analysis was performed and PCR products were separated by gel electrophoresis. In order to ensure that the primers obtained were specific for the *P gingivalis* bacterium and not simply for generic bacterial sequences, samples of *Salmonella typhimurium* and *Escherichia coli* were used as negative controls in all of the PCR reactions. In every instance, PCR analysis failed to generate a positive band when either *S typhimurium* or *E coli* was used. Additionally, samples taken from the original vial of *P gingivalis* obtained from ATCC served as a positive control for these experiments to ensure that the PCR primers were capable of detecting the *P gingivalis* bacterium. In every instance, the positive control generated a band following gel electrophoresis of the PCR products. Finally, PCR analysis of every monthly sample that was taken throughout the experimental period generated a positive band detecting the presence of *P gingivalis*, following gel electrophoresis (**Figure 8**).

Figure 8: Quantification of *Porphyromonas gingivalis in vitro.*

P gingivalis strain 381 was grown in anaerobic broth under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37°C. Samples of *P gingivalis* were taken on a monthly basis throughout the entire experimental period in order to ensure successful growth of the bacterium. Bacterial samples were pelleted by centrifugation at 4000 rpm for 4 minutes. The supernatant was removed, and DNA was extracted from the pelleted bacteria overnight at 55°C. In order to confirm the presence of *P gingivalis*, PCR analysis was performed using primers specific for the bacterium. This figure depicts a representative image of PCR products separated by gel electrophoresis on a 1.5% agarose gel. Presence of a PCR product confirms that the sample is *P gingivalis*. *S typhimurium* and *E coli* were used as negative controls in this experiment to ensure the PCR primers were specific to unique *P gingivalis* sequences and not generic bacterial sequences. A sample of *P gingivalis* isolated from the original vial of bacteria obtained from ATCC served as a positive control for these experiments.


E coli
S typhimurium
P gingivalis
Sample

In order to determine the concentration of *P gingivalis*, the bacteria was allowed to grow in anaerobic broth until the optical density reached 0.8 at 660 nm. Serial dilutions were then plated on anaerobic agar plates and incubated in an anaerobic environment. Colonies of *P gingivalis* were visible on the agar plates within 3-5 days post-inoculation. Growth of the bacteria on the anaerobic agar plates indicated that at an optical density of 0.8 at 660 nm the concentration of *P gingivalis* was 10^9 CFU/mL. This concentration was used to determine the volume of *P gingivalis* used in both the *in vitro* studies, as well as the oral inoculation of mice for the *in vivo* atherosclerosis studies.

5.2 *In vitro* studies

5.2.1 *P gingivalis* is recognized and internalized by macrophages

Since the macrophage is a critical component in the formation of early atherosclerotic lesions we made use of electron microscopy to examine the relationship between this critical component of atherosclerosis and the *P gingivalis* bacterium. In order to do so, we tested the ability of mouse peritoneal macrophages to recognize and take up *P gingivalis*, using an *in vitro* invasion assay outlined by Dorn *et al* (Dorn *et al.*, 1999). The purpose of this experiment was 2-fold: (1) to determine the relationship between macrophages and *P gingivalis* and (2) to determine if this relationship could be visualized using electron microscopy.

Peritoneal macrophages isolated from *apoE* deficient mice were exposed to *P gingivalis* at a multiplicity of infection of 50 for 90 minutes under aerobic conditions. Following exposure to the live bacterium, macrophages were examined by electron microscopy for evidence of *P gingivalis* invasion (**Figure 9**). Compared to uninfected mouse peritoneal macrophages, numerous *P gingivalis* bacteria were apparent inside the

macrophages infected with the bacterium, indicating that macrophages are able to recognize and internalize *P gingivalis* (**Figure 9A & B**). After the 90 minute exposure, *P gingivalis* was mostly visible within the cytoplasm of the macrophages, enclosed within membrane bound endocytic vacuoles. Similar to results seen by Dorn *et al* upon infection of human coronary artery cells (Dorn *et al.*, 1999), in one of the electron microscopy pictures *P gingivalis* appears to be dividing within the macrophage (**Figure 9B**), indicating the possibility that this bacterium may remain metabolically active during up-take by the macrophages and be able to persist within the macrophage for at least a short period of time. In macrophages that were not incubated with *P gingivalis*, there was no evidence of bacterial internalization (**Figure 9C**).

5.2.2 Exposure to live *P gingivalis* promotes caspase-1 activation

Several studies have implied that *P gingivalis* is capable of activating the caspase-1 enzyme *in vitro* by monitoring the expression levels of IL-1β, a cytokine that requires caspase-1 activity for activation (Zhou *et al.*, 2005). Since one of the central topics of the current study is the ability of *P gingivalis* to activate caspase-1, it was essential that the relationship between *P gingivalis* and caspase-1 activation be examined. As such, an *in vitro* assay was used to directly assess the ability of *P gingivalis* to activate caspase-1. A study conducted by Mariathasan *et al* (Mariathasan *et al.*, 2004) demonstrated the ability of *Salmonella typhimurium* to directly activate the caspase-1 enzyme within murine macrophages, therefore this bacterium was used as a positive control in these studies.

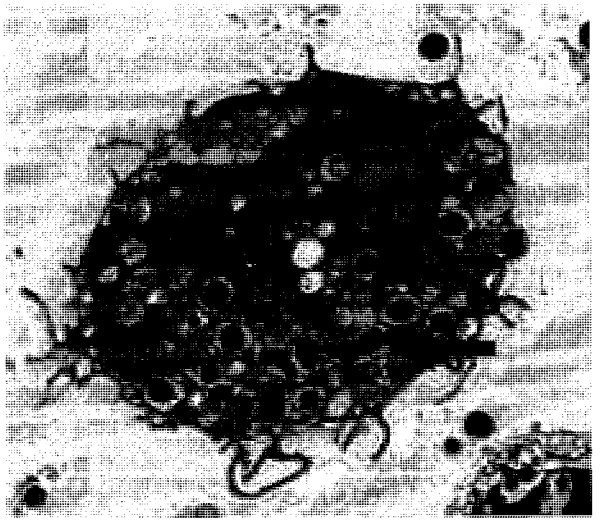
Caspase-1 is constitutively expressed as a 45kDa inactive proenzyme, requiring proteolytic cleavage to generate a heterodimer, which contains a 10-kDa (p10) subunit and a 20-kDa (p20) subunit (Martinon and Tschopp, 2004). The active caspase-1 enzyme

Figure 9: Electron microscopy of *P gingivalis* invasion of macrophages.

Thioglycollate-elicited peritoneal macrophages were harvested from male *apoe*$^{-/-}$ mice by peritoneal lavage four days after injection with 4% thioglycollate into the peritoneal cavity. Macrophages were then plated in a 12-well culture plate at a concentration of 1 x 10^6 cells/mL and exposed to 10^9 bacterial cells of *P gingivalis* at 37°C for 90 minutes under aerobic conditions (5% CO_2). In order to more closely mimic *in vivo* conditions, the bacteria were not centrifuged onto the cells to promote contact. Following 90 minutes of exposure, the cells were washed, fixed in 1.6% gluteraldehyde, and prepared for analysis by electron microscopy. **(A)** *apoe*$^{-/-}$ macrophage in the presence of *P gingivalis*. Numerous *P gingivalis* bacteria are apparent inside the macrophage apparantly enclosed within membrane bound endocytic vacuoles. **(B)** In this image, internalized *P gingivalis* bacteria appear to be dividing within the macrophages (indicated by an arrow). **(C)** *apoe*$^{-/-}$ macrophage in the absence of *P gingivalis*.

A.

B.



C.

consists of two of the p10/p20 heterodimers, with the active site of the enzyme being located on the p10 subunit (Martinon and Tschopp, 2004). Since the active site of the enzyme is located on the p10 subunit, Western blots were probed using antibodies directed against the p10 subunit of the active caspase-1 enzyme.

Mariathasan *et al* (Mariathasan *et al.*, 2004) previously showed that incubation of peritoneal macrophages with *Salmonella typhimurium* generated an active caspase-1 enzyme (**Figure 10A**). For the purposes of this study *S typhimurium* was the positive control for the activation of caspase-1. Caspase-1 activation is an autocatalytic process that occurs through the generation of a series of intermediates produced by the proteolytic cleavage of four internal peptide bonds within the 45 kDa pro-enzyme. These intermediates in caspase-1 activation have molecular masses of 45, 36, 27, 25, 23, 14, and 12 kDa respectively (Ramage *et al.*, 1995). The bands at 25, 23, and 12 kDa were previously identified as the p24, p20 and p 10 subunits respectively (Ramage *et al.*, 1995). The band at 36 kDa corresponds to the cleavage occurring at the C-terminal end of the pro-enzyme, whereas the band at 14 kDa consists of the elongated version of the p10 subunit (Ramage *et al.*, 1995). The 45 kDa band was shown to contain the full-length pro-enzyme, while the 27 kDa band was identified as the N-terminal domain of the pro-enzyme (Ramage *et al.*, 1995). For the purposes of this study, it is of particular importance to focus attention on the band seen at 10kDa in the blot as this corresponds to the p10 subunit of the enzyme and indicates that the caspase-1 enzyme has been activated (**Figure 10B**).

In order to determine the amount of time needed to generate a maximally active caspase-1 enzyme following exposure to *P gingivalis,* a time-course monitoring caspase-

1 cleavage was carried out. Mouse peritoneal macrophages isolated from *apoe* deficient mice, plated at a concentration of 1 x 10^6 cells/mL, were exposed to *P gingivalis* at a multiplicity of infection of 50 for various time periods (8, 10, 12, 14, 16 minutes are shown in **Figure 10B**). Cells were then lysed and the lysates were subjected to Western blot analysis using an antibody specific for the p10 subunit of caspase-1. Following macrophage exposure to *P gingivalis*, the p10 band is generated, matching the p10 band generated by the positive control, and indicating that an active caspase-1 enzyme has been generated. Maximum caspase-1 cleavage was observed in macrophages following 14 minutes of exposure to *P gingivalis* **(Figure 10B).**

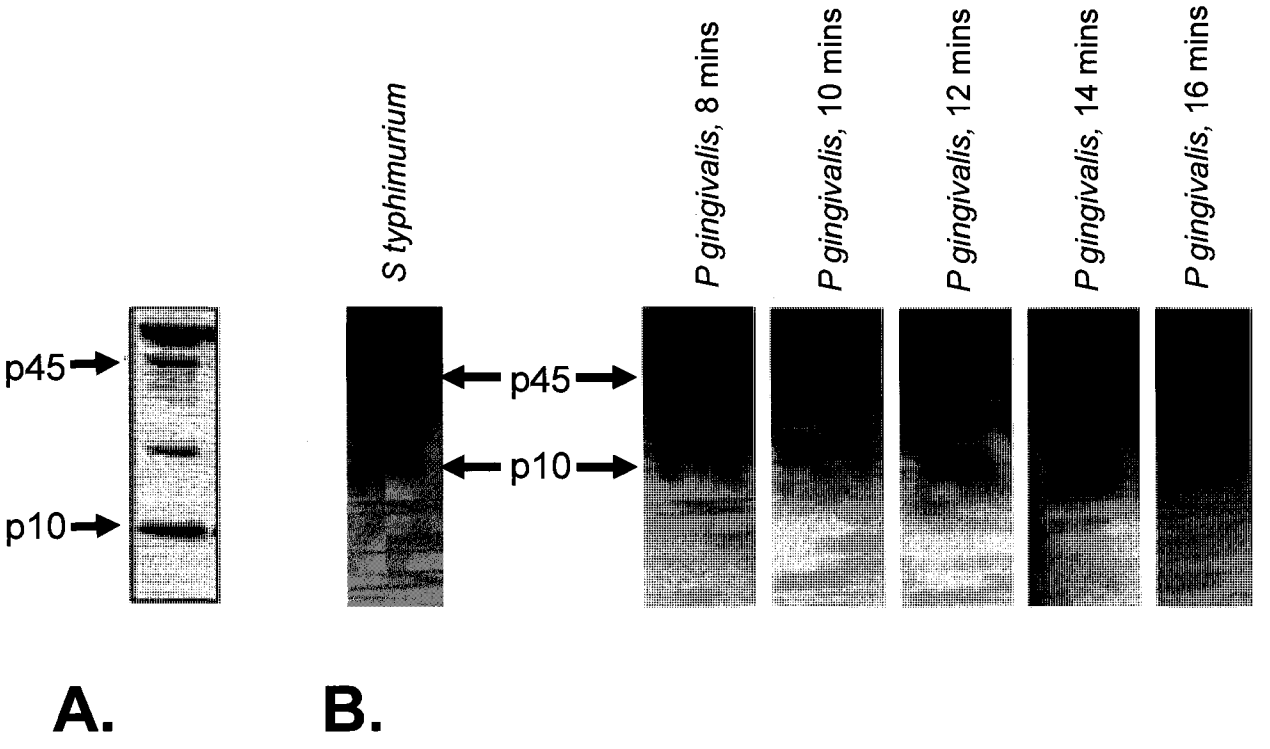
5.2.3 Differential cytokine expression of macrophages versus foam cells exposed to live *P gingivalis*

Zhou *et al* (Zhou *et al.*, 2005) have investigated cytokine profiles generated by macrophages versus foam cells following infection with live *P gingivalis in vitro*. Thioglycollate-elicited peritoneal macrophages from *apoe*$^{-/-}$ mice were exposed to oxLDL in order to generate foam cells, while macrophages untreated with lipoproteins were used as a control for the assay. Experiments were run three times in duplicate. Foam cell formation was qualitatively monitored through an *in vitro* foam cell assay. After macrophages were cultured in the presence of 100 μg/mL of oxLDL, they were stained with Oil Red O to detect cytoplasmic cholesterol ester droplets. Compared to untreated macrophages, analysis revealed that most macrophages treated with oxLDL developed into lipid laden foam cells (**Figure 11**). This was a key step in the assay as it demonstrates that the method for generating foam cells was successful, hence the results presented regarding differential cytokine expression between macrophages and foam cells does in fact evaluate the behaviours of these two cell types.

Figure 10: Activation of caspase-1 by *P gingivalis*.

Peritoneal macrophages isolated from *apoe*$^{-/-}$ mice were exposed to *S typhimurium* or *P gingivalis* at an MOI of 50. Membranes were probed with an antibody directed towards the active p10 subunit of the caspase-1 enzyme. **(A)** Results from Mariathasan *et al* (Reprinted with permission from Macmillan Publishers Ltd: Nature, Mariathasan *et al* (Mariathasan *et al.*, 2004), copyright 2004) where mouse peritoneal macrophages incubated with *S typhimurium* generated an active caspase-1 enzyme (indicated by p10). **(B)** Mouse peritoneal macrophages were exposed to *P gingivalis* for various time periods (8, 10, 12, 14, and 16 minute time points are shown in the figure). Cell lysates were then subjected to Western blot using an antibody directed against the p10 subunit of caspase-1. The active caspase-1 enzyme was generated following exposure to *P gingivalis*, as indicated by p10, with maximal caspase-1 activation occurring following 14 minutes of exposure to *P gingivalis*. *S typhimurium* was used as a positive control for our study.

(p45: 45kDa inactive pro-enzyme; p10: 10kDa active caspase-1 enzyme)

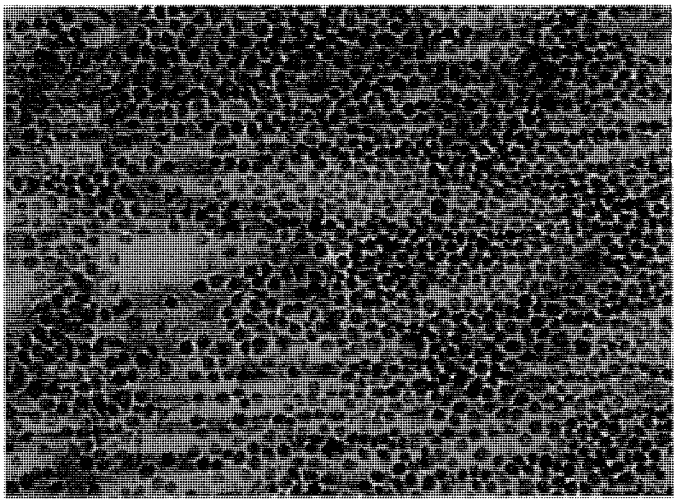

p45
p10
S typhimurium
P gingivalis, 8 mins
P gingivalis, 10 mins
P gingivalis, 12 mins
P gingivalis, 14 mins
P gingivalis, 16 mins
p45
p10
A.
B.

Following the successful generation of foam cells by treatment of peritoneal macrophages with 100μg/mL of oxLDL, cells were then exposed to live *P gingivalis* at an MOI of 50 for 24 hours. Control macrophages were cultured in the absence of oxLDL for the same time period prior to incubation with *P gingivalis.* Experiments were run three times in duplicate. Culture media was then subject to a cytokine antibody array which detects the expression of 62 different cytokines (**Figure 12**). **Table 1** summarizes the cytokines that were analysed, along with their properties and functions. Following analysis of culture media it was determined that foam cells that had been infected with the *P gingivalis* bacterium generated a different cytokine profile than macrophages that had been exposed to the bacterium. **Table 2** summarizes the changes in cytokine expression in macrophages and foam cells following treatment with *P gingivalis.*

Figure 11: Foam cell formation in *apoe*$^{-/-}$ macrophages.

To ensure that oxLDL induces the transformation of macrophages to foam cells, murine *apoe*$^{-/-}$ macrophages were cultured in the presence of 100 μg/mL of oxLDL, and were stained with Oil Red O to detect macrophages containing cytoplasmic cholesterol ester droplets. The presence of these cytoplasmic cholesterol ester droplets is characteristic of foam cells. **(A)** Macrophages with no exposure to oxLDL. **(B)** Incubation with oxLDL induced macrophage foam cell formation.

A.



B.

5.3 In vivo studies

5.3.1 Plasma lipoprotein and cholesterol levels

Plasma lipoprotein and cholesterol levels were measured for mice in all groups following tissue collection at the endpoint of this study. Compared to *apoe*$^{-/-}$ mice that were caspase-1$^{+/+}$, deficiency in endogenous caspase-1 in the *apoe*$^{-/-}$ strain did not affect the serum total cholesterol levels. Analysis by size exclusion chromatography also confirmed that there were no differences in lipoprotein-cholesterol profiles between caspase-1$^{+/+}$ and caspase-1$^{-/-}$ animals (data not shown). Furthermore, no significant differences were reported in either serum total cholesterol levels or in the lipoprotein-cholesterol profiles in control versus *P gingivalis* inoculated mice. In all of the mice, the majority of circulating cholesterol was contained within the lipoprotein fractions consisting of chylomicrons, VLDL and their remnants (data not shown).

5.3.2 Body weights

Body weights were recorded for animals in all groups throughout the experimental period. Compared to *apoe*$^{-/-}$ mice that were competent for caspase-1, deficiency in endogenous caspase-1 did not affect body weight recorded at the end of the experimental period in either male or female mice. Moreover, oral challenge with *P gingivalis* did not have any affect on body weight in either male or female caspase-1$^{+/+}$ versus caspase-1$^{-/-}$ mice (data not shown).

Figure 12: ***In vitro*** **cytokine antibody array detecting 62 cytokines.**

Foam cells generated from peritoneal macrophages isolated from *apoe*$^{-/-}$ mice were exposed to live *P gingivalis* at an MOI of 50:1 for 24 hours. Untreated macrophages were used as a control. Cell culture media was subjected to a cytokine antibody array. **(A)** Each cytokine is represented by duplicate spots on the locations shown. **(B)** Cytokine antibody array following incubation with cell culture media, clockwise from top left: macrophages, foam cells, macrophages exposed to *P gingivalis*, foam cells exposed to *P gingivalis*.

A.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	Blank	Axl	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
2	POS	POS	NEG	NEG	Blank	Axl	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
3	Eotaxin-2	Fas Ligand	Fractakine	GCSF	GM-CSF	IFN-r	IGFBP-3	IGFBP-5	IGFBP-6	IL-1a	IL-1b	IL-2	IL-3	IL-3Rb
4	Eotaxin-2	Fas Ligand	Fractakine	GCSF	GM-CSF	IFN-r	IGFBP-3	IGFBP-5	IGFBP-6	IL-1a	IL-1b	IL-2	IL-3	IL-3Rb
5	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13	IL-17	KC	Leptin R	LEPTIN(OB)	LIX	L-Selectin
6	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13	IL-17	KC	Leptin R	LEPTIN(OB)	LIX	L-Selectin
7	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1a	MIP-1r	MIP-2	MIP-3b	MIP-3a	PF-4	P-Selectin	RANTES	SCF
8	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1a	MIP-1r	MIP-2	MIP-3b	MIP-3a	PF-4	P-Selectin	RANTES	SCF
9	SDF-1a	TARC	TCA-3	TECK	TIMP-1	TNF-a	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	Blank	Blank	POS
10	SDF-1a	TARC	TCA-3	TECK	TIMP-1	TNF-a	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	Blank	Blank	POS

B.

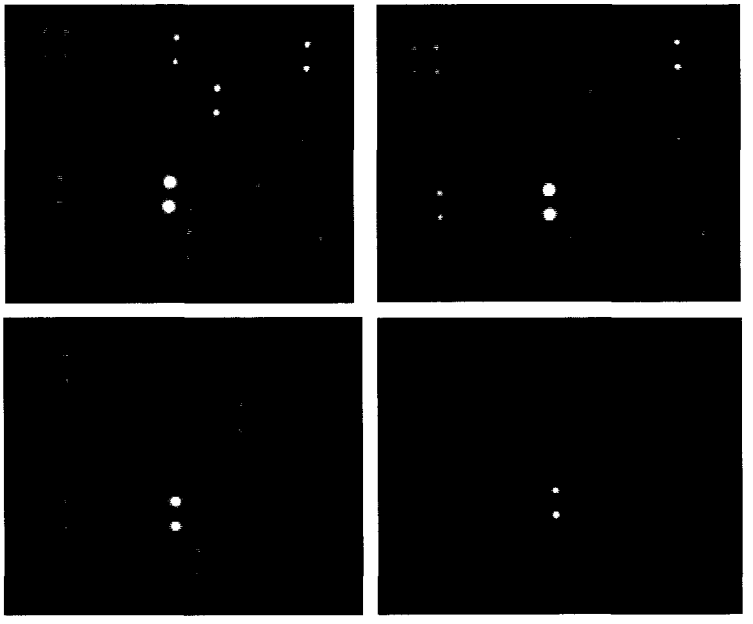


Table 1. Summary of cytokines measured using RayBiotech Cytokine Antibody Array III, and their associated properties.

CYTOKINE	PROPERTIES
axl	• a protein tyrosine kinase
BLC	• B lymphocyte chemoattractant • promotes migration of T cells and macrophages
CD30/TNFRSF8	• cell surface antigen • expressed in B and T cells
CD30L	• CD-30 ligand • type 2 membrane protein • enhances proliferation of T cells
CD40	• cell surface antigen • expressed on B-lymphocytes, T-cells and monocytes,
CRG-2	• chemotactic cytokine • synthesis induced by interferons and bacterial LPS
CTACK	• cutaneous T-cell attracting chemokine • attracts circulating memory T-cells • stimulated by TNF-alpha, IL-1beta
CXCL16	• expressed on the surface of antibody-presenting cells including CD19+ B-cells, CD14+ monocytes and macrophages
Eotaxin	• belongs to platelet factor 4 chemokine family • potent stimulator of eosinophils
Eotaxin-2	• chemokine • induces chemotaxis of eosinophils and basophils
FAS ligand	• membrane bound protein
Fractalkine	• chemokine • chemoattractant for T cells and monocytes
G-CSF	• granulocyte colony stimulating factor
GM-CSF	• granulocyte-macrophage colony stimulating factor
IFN-gamma	• produced by T-cells, and NK cells • modulates immune responses
IGF-BP-3	• insulin-like growth factor binding protein 3 • major IGF-BP in serum of humans and animals • act as carrier protein for IGF
IGF-BP-5	• insulin-like growth factor binding protein 5 • major IGF-BP in kidney of humans and animals • act as carrier protein for IGF
IGF-BP-6	• insulin-like growth factor binding protein 6 • major IGF-BP in cerebrospinal of humans and animals • act as carrier protein for IGF
IL10	• produced by monocytes, TH2-cells, mast cells • inhibits Th1 cytokine production
IL12-p70	• produced by macrophages • involved in NK cell stimulation and Th1 induction
IL17	• induces the production of inflammatory cytokines

IL1-alpha	• pro-inflammatory cytokine involved in immune defence of infection • produced by macrophages, monocytes and dendritic cells • predominantly membrane bound
IL1-beta	• pro-inflammatory cytokine involved in immune defence of infection • produced by macrophages, monocytes and dendritic cells • predominantly secreted
IL2	• produced by TH1 cells • stimulates growth and differentiation of T cell
IL3	• produced by T cells • stimulates bone marrow cells
IL4	• produced by TH2 cells, memory CD4+ cells • involved in proliferation of B cells and development of T cells and mast cells
IL5	• produced by TH2 cells • role in differentiation of B cells, eosinophil production and IgA production
IL6	• produced in macrophages and TH2 cells • induces acute phase response
IL9	• produced by T-cells, specifically by CD4+ helper cells • stimulates mast cells
KC	• keratinocyte chemoattractant • involved in chemotaxis and cell activation of neutrophils
Leptin	• homolog of murine obese gene • expressed in white adipose tissue and brown adipose tissue • inhibits food intake and energy expenditure
Leptin R	• leptin receptor
LIX	• LPS induced CXC chemokine • Expression induced by bacterial LPS in fibroblasts
L-Selectin	• antigen expressed on B cells, T cells, monocytes, neutrophils, eosinophils, NK cells
Lymphotactin	• chemoattractant for T cells
MCP-5	• monocyte chemoattractant protein • chemoattractant for peripherial blood monocytes
M-CSF	• macrophage colony stimulating factor • produced by monocytes, granulocytes, endothelial cells, and fibroblasts
MIG	• monokine induced by gamma interferon • expression induced by IFN gamma in macrophages
MIP-1-alpha	• macrophage inflammatory protein 1 alpha • produced by macrophages following stimulation with bacterial endotoxins
MIP-1-gamma	• novel murine chemokine
MIP-2	• chemotactic for neutrophilic granullocytes
MIP-3-alpha	• Expressed in lymph nodes, appendix, peripheral blood leukocytes, fetal liver, fetal lung
MIP-3-beta	• expressed in lymph nodes, thymus, appendix
PF4	• platelet factor 4 • syntehsized in megakaryocytes and platelets • chemotactic for inflammatory cells such as neutrophils and monocytes
P-Selectin	• antigen expressed on platelets, megakaryocytes, endothelial cells
RANTES	• regulated upon activation, normal T-cell expressed, and secreted • chemotactic for T cells, eosinophils, basophils, and plays a role in recruiting leukocytes into inflammatory sites

SCF	• stem cell factor • important for the survival, proliferation, and differentiation of hematopoetic stem cells
SDF-1-alpha	• stromal cell derived factor 1-alpha • chemotactic for lymphocytes
TARC	• thymus and activation regulated chemokine • expressed in thymic dendritic cells, lymph node dendritic cells, and CD11+ cells in the lung
TCA-3	• T-cell activation 3 • produced by T cells after activation
TECK	• thymus expressed chemokine • produced by thymic dendritic cells • chemotactic for macrophages, dendritic cells, and thymocytes
TIMP-1	• tissue inhibitor of metalloproteinase • inhibits activity of matrix malleoproteinases
TNF-alpha	• tumor necrosis factor alpha • cytokine involved in systemic inflammation
TPO	• Thrombopoietin • stimulates the production and differentiation of megakaryocytes
VCAM-1	• vascular cell adhesion molecule-1 • promotes adhesion of lymphocytes, monocytes, eosinophils and basophils
VEGF	• vascular endothelial growth factor • stimulates the migration of monocytes and macrophages

Table 2. Changes in cytokine expression of macrophages and foam cells following exposure to *P gingivalis*.

CYTOKINE	Macrophages vs Macrophages exposed to *P gingivalis*	Foam Cells vs Foam cells exposed to *P gingivalis*
axl	↓	↑
BLC	↓	↑
CD30/TNFRSF8	↓	↑
CD30L	↓	↑
CD40	↓	↑
CRG-2	↓	↑
CTACK	↓	↑
CXCL16	↓	↑
Eotaxin	↓	↑
Eotaxin-2	↑	↓
FAS ligand	↑	↓
Fractalkine	↑	↓
G-CSF	↓	↑
GM-CSF	↓	↑
IFN-gamma	↓	↑
IGF-BP-3	↓	↑
IGF-BP-5	↓	↑
IGF-BP-6	↓	↑
IL10	↓	↑
IL12-p70	↓	↑
IL17	↓	↑
IL1-alpha	↑	↑
IL1-beta	↓	↑
IL2	↓	↑
IL3	↑	↓
IL4	↔	↓
IL5	↑	↓
IL6	↑	↓
IL9	↓	↑

KC	↓	↑
Leptin	↓	↑
Leptin R	↓	↑
LIX	↓	↓
L-Selectin	↔	↓
Lymphotactin	↓	↑
MCP-5	↓	↑
M-CSF	↑	↓
MIG	↓	↑
MIP-1-alpha	↓	↑
MIP-1-gamma	↓	↑
MIP-2	↓	↑
MIP-3-alpha	↓	↓
MIP-3-beta	↓	↑
PF4	↓	↑
P-Selectin	↓	↑
RANTES	↓	↑
SCF	↓	↑
SDF-1-alpha	↓	↑
TARC	↓	↑
TCA-3	↓	↑
TECK	↓	↑
TIMP-1	↓	↑
TNF-alpha	↓	↑
TPO	↓	↑
VCAM-1	↓	↑
VEGF	↓	↑

5.3.3 Oral infection with *P gingivalis* promotes alveolar bone loss

Periodontal disease encompasses a group of infections that affect the supporting tissues of the teeth, where destruction of the connective tissue and alveolar bone can lead to tooth loss. Alveolar bone loss is therefore one of the hallmarks of periodontal disease. To determine the degree of local periodontal infection and destruction resulting from an oral challenge with *P gingivalis*, mandibles were dissected from both caspase-$1^{-/-}$ x $apoe^{-/-}$ and caspase-$1^{+/+}$ x $apoe^{-/-}$ mice following euthanasia and alveolar bone loss was measured. For each mouse, alveolar bone loss is defined as the total area between the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) for a total of 6 mandibular posterior teeth (**Figure 13**). Both female and male animals given the oral challenge of *P gingivalis* displayed significantly increased bone loss compared with animals not challenged with the bacterium (**Figure 14**, **Figure 15** respectively). Analysis revealed that there was a statistically significant 25% and 34% increase in bone loss in caspase-$1^{+/+}$ male and female mice respectively, challenged with the bacterium compared to those that did not receive the oral challenge (blue bars versus orange bars). In mice deficient in endogenous caspase-1, there was a 43% and 47% increase in bone loss in male and female mice respectively, challenged with the bacterium as compared with animals that did not receive the oral challenge (pink versus green bars). It is important to note that no significant difference in alveolar bone loss was observed between caspase-$1^{+/+}$ and caspase-$1^{-/-}$ mice orally challenged with *P gingivalis* (blue versus pink bars)

Interestingly, control mice not challenged with the *P gingivalis* bacterium, animals that were competent for the caspase-1 enzyme displayed significantly greater 13% and 14% increase in alveolar bone loss in male and female mice respectively, than

Figure 13: Evaluation of alveolar bone loss in mice.

Evaluation of *P gingivalis* induced periodontal destruction was determined by measurement of alveolar bone loss in the mice. Following oral challenge with *P gingivalis*, mandibles were removed from the mice under the dissecting microscope, and using forceps and a scalpel, the lingual gingival tissue was removed. Following removal of the gingival tissue, the mandibles were exposed to 3% H_2O_2 overnight and then mechanically defleshed. The lingual surface of the jaws was photographed using a digital CoolSNAP *cf* camera. For each mouse, alveolar bone loss was defined as the total area between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC) for a total of 6 mandibular posterior teeth. Analysis was performed using Image-Pro software. **(A)** Image of hemi-sected mandible isolated from a control mouse. **(B)** Image of a hemi-sected mandible isolated from a mouse following oral challenge with *P gingivalis*.

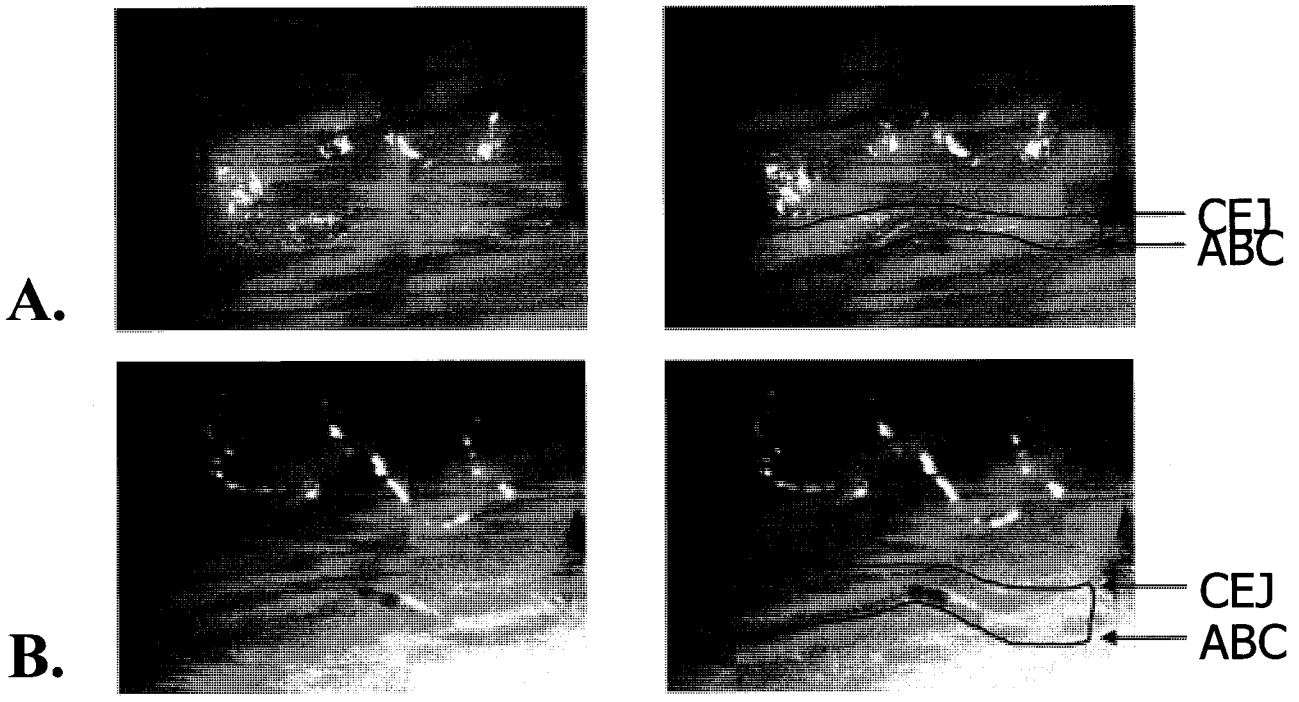

A.
CEJ
ABC
B.
CEJ
ABC

Figure 14: Oral infection enhances alveolar bone loss in male mice.

Alveolar bone loss in male mice that are either caspase-$1^{+/+}$ *apoe*$^{-/-}$ or caspase-$1^{-/-}$ *apoe*$^{-/-}$ that have been orally challenged with *P gingivalis* (PG) versus those not challenged with the bacterium (control). For each mouse, alveolar bone loss is defined as the total area between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC) for a total of 6 mandibular posterior teeth. Data shown is ± SEM. (N= 14 for casp$1^{-/-}$ *apoe*$^{-/-}$ Pg; N= 16 for casp$1^{+/+}$ *apoe*$^{-/-}$, Pg; N= 14 for casp$1^{-/-}$ *apoe*$^{-/-}$, Control; N= 16 for casp$1^{+/+}$ *apoe*$^{-/-}$, Control).

#, $p = 0.015$; ‡, $p \leq 0.0001$; §, $p \leq 0.0001$

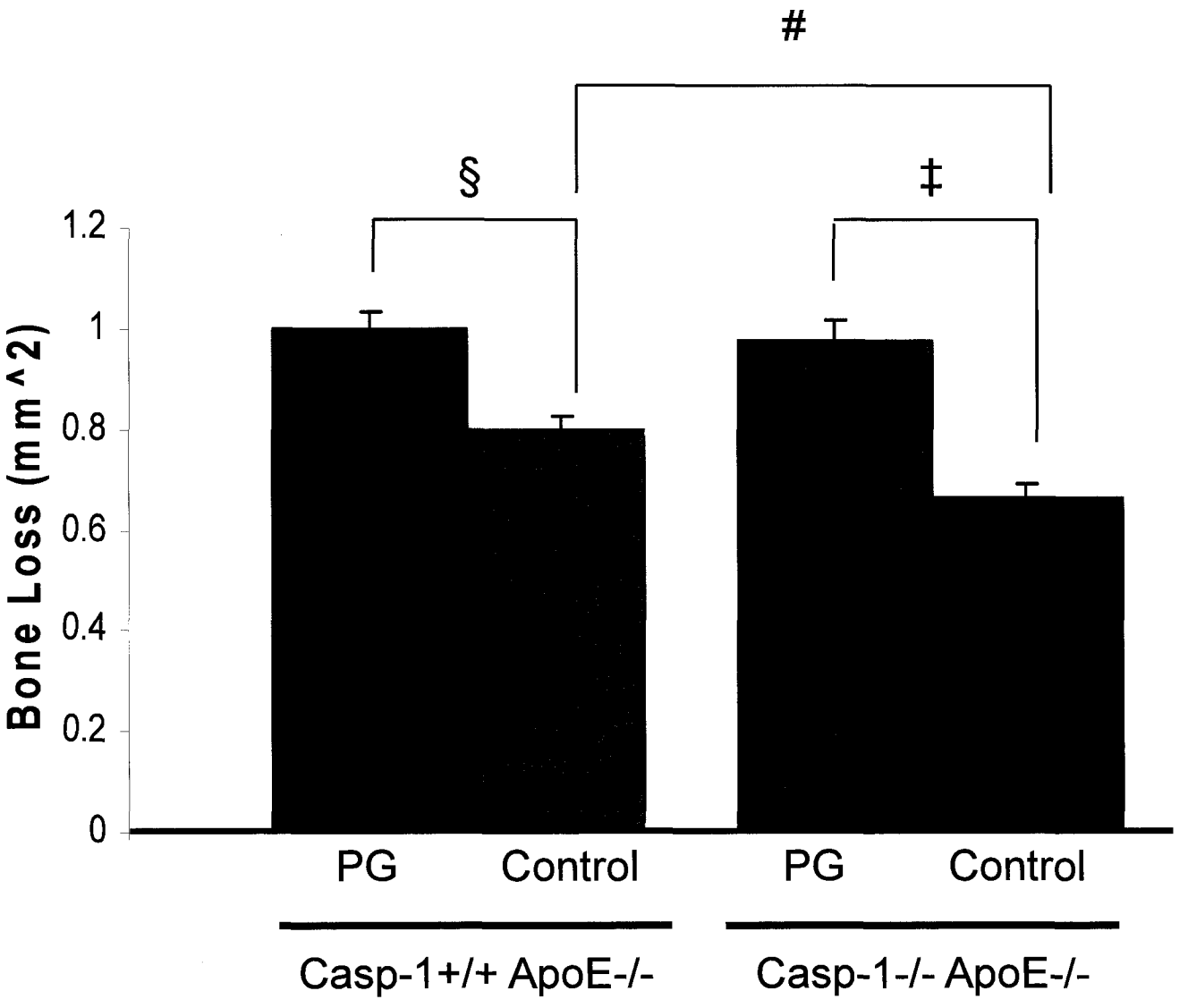
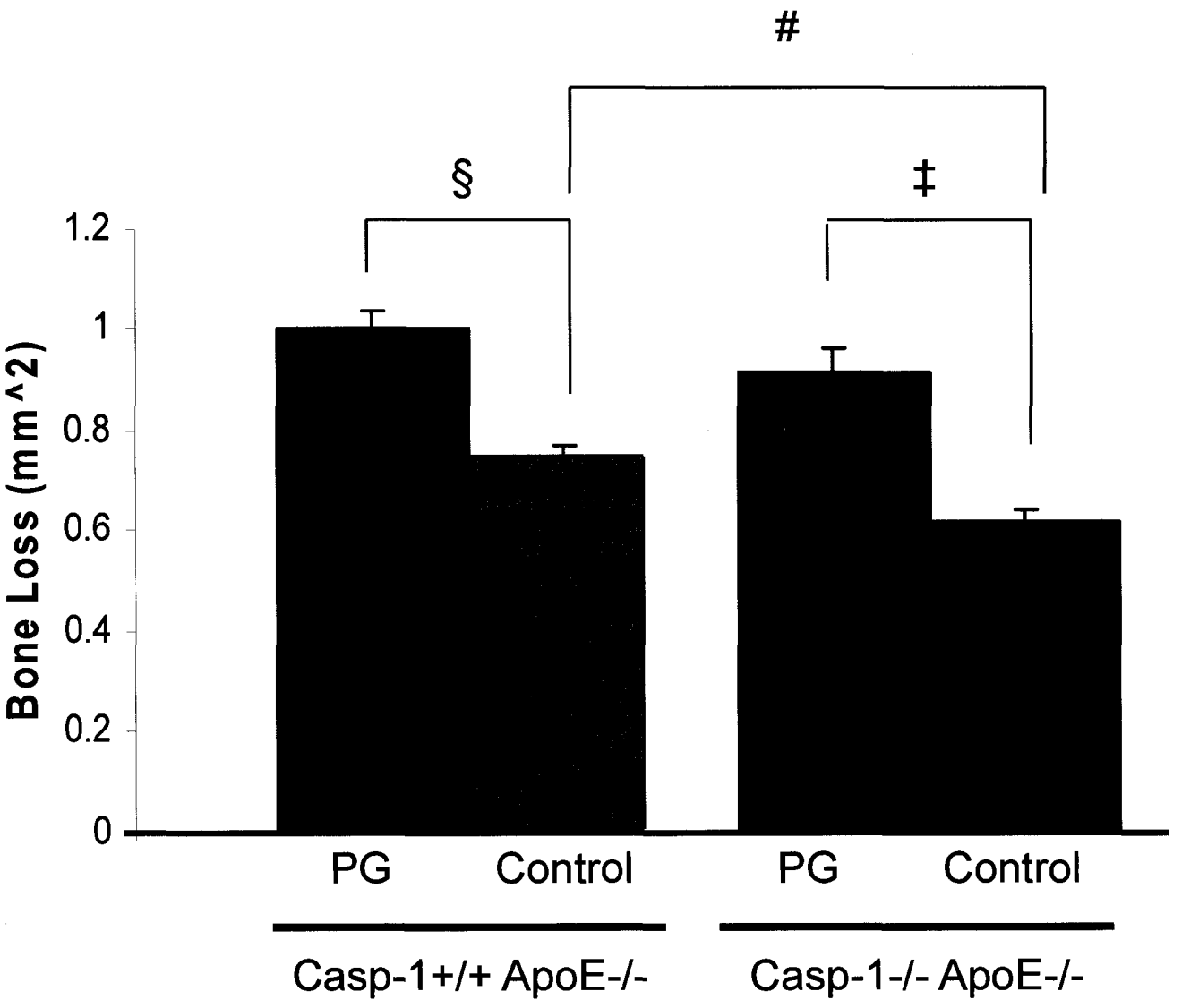

#
§
‡
1.2
1
0.8
0.6
0.4
0.2
0
Bone Loss (mm^2)
PG
Control
PG
Control
Casp-1+/+ ApoE-/-
Casp-1-/- ApoE-/-

Figure 15: Oral infection enhances alveolar bone loss in female mice.

Alveolar bone loss in female mice that are either caspase-1 $^{+/+}$ *apoe*$^{-/-}$ or caspase-1$^{-/-}$ *apoe*$^{-/-}$ that have been orally challenged with P gingivalis (PG) versus those not challenged with the bacterium (control). For each mouse, alveolar bone loss is defined as the total area between the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) for a total of 6 mandibular posterior teeth. Data is shown ± SEM. (N= 15 for casp1-/- *apoe*$^{-/-}$, Pg; N= 15 for casp1+/+ *apoe*$^{-/-}$, Pg; N= 15 for casp1-/- *apoe*$^{-/-}$, Control; N= 15 for casp1+/+ *apoe*$^{-/-}$, Control).

#, $p = 0.016$; ‡, $p \leq 0.0001$; §, $p \leq 0.0001$


#
§
‡
1.2
1
0.8
0.6
0.4
0.2
0
Bone Loss (mm^2)
PG
Control
PG
Control
Casp-1+/+ ApoE-/-
Casp-1-/- ApoE-/-

mice deficient in caspase-1 (**Figure 14**, **Figure 15** respectively; orange versus green bars).

5.3.4 *P gingivalis* promotes lesion development in caspase-$1^{+/+}$ mice

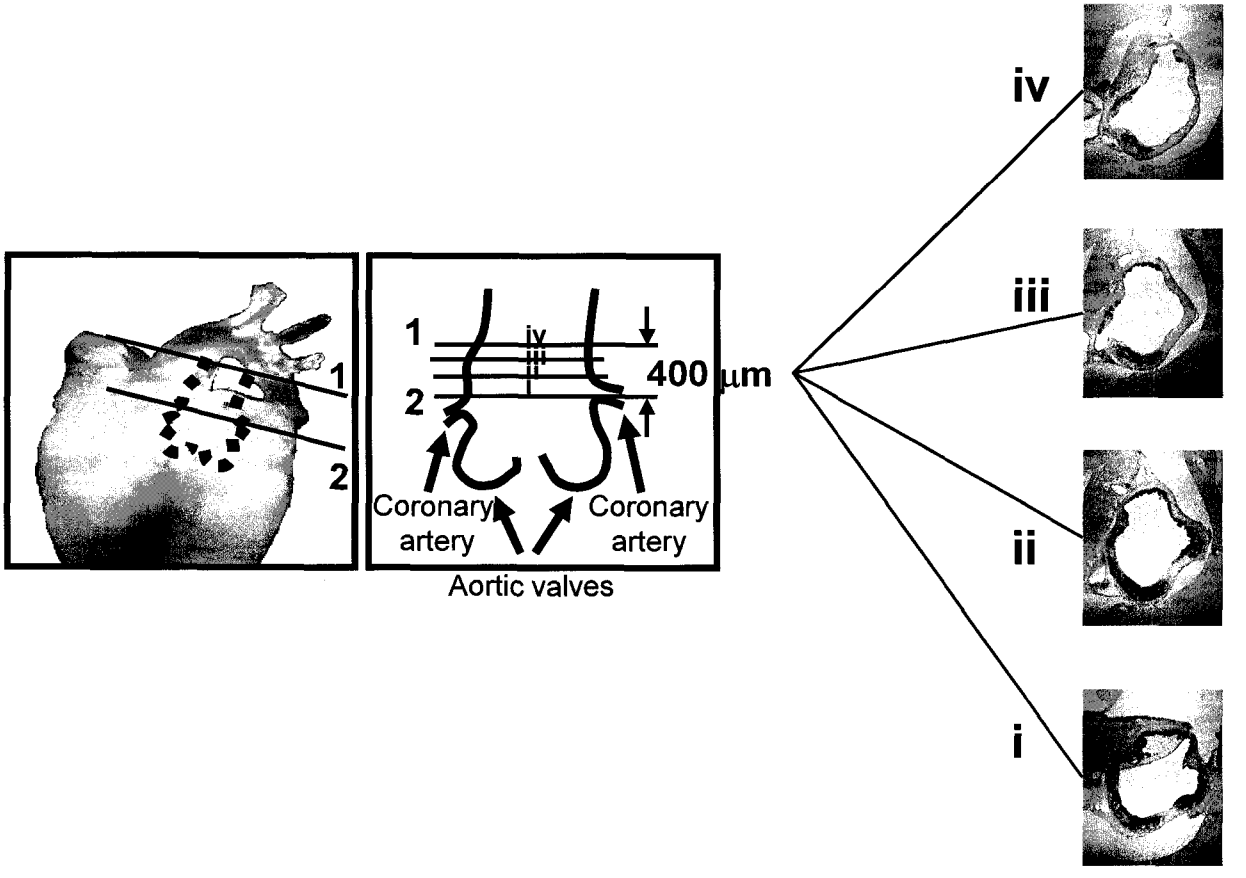
In a study conducted by Lalla *et al* (Lalla *et al.*, 2003), *apoe*$^{-/-}$ male mice that were given an oral challenge of *P gingivalis* exhibited a significant increase in atherosclerotic lesion development as compared to mice not infected with the bacterium. Additionally, preliminary studies conducted by our laboratory demonstrated that loss of endogenous caspase-1 expression significantly decreased atherosclerotic lesion size.

In the present study, 6-week old atherosclerosis susceptible *apoe*$^{-/-}$ mice that were either competent (+/+) or deficient (-/-) for the endogenous caspase-1 enzyme were orally challenged with 10^{10} bacterial cells of *P gingivalis* over a 3 week period, for a total of 15 inoculations. At 17 weeks of age, tissues were collected from both the experimental and control mice, and atherosclerotic lesion development was assessed in the ascending aorta. To determine atherosclerotic lesion size within the ascending aorta, the mean lesion area was derived from 4 serial Sudan IV stained sections that were cut 10μm thick and collected 100μm apart over a 1mm segment of the aortic root (**Figure 16**).

Analysis of the control mice lend further support to the preliminary findings, where loss of endogenous caspase-1 expression significantly decreased lesion size in both male and female mice that had not been challenged with *P gingivalis* (**Figure 17**, **Figure 18**, $p = 0.026$, $p = 0.043$ respectively; orange triangle versus green x). Analysis also indicated that oral challenge with the *P gingivalis* bacterium significantly increased atherosclerotic lesion development in male caspase-$1^{+/+}$ mice as compared to male

Figure 16: Methodology of atherosclerotic lesion analysis.

A diagram of the mouse heart showing the region of the ascending aorta where we collect serial sections for the measurement of atherosclerotic lesion size. To determine atherosclerotic size within the ascending aorta, the mean lesion area was be derived from 4 serial Sudan IV stained sections that were cut 10 μm thick, and collected 100 μm apart over a 1 mm segment of the aortic root.


1
2
1
2
iv
iii
ii
i
400 µm
Coronary artery
Coronary artery
Aortic valves
iv
iii
ii
i

animals not given the oral challenge (**Figure 17**, $p = 0.042$; blue versus orange bars). There was a statistically significant 105% increase in mean atherosclerotic lesion development in male caspase-$1^{+/+}$ *P gingivalis*-infected animals as compared with controls. Furthermore, no difference in lesion development was observed in male caspase-$1^{-/-}$ mice orally challenged with *P gingivalis* compared with those not given the oral challenge (pink versus green bars).

Tissue analysis in the aortic root of the female mice revealed there was no significant difference in lesion development in caspase-$1^{-/-}$ mice orally challenged with the *P gingivalis* bacterium as compared with those mice not given the oral challenge (**Figure 18**, pink versus green bars). Although there appears to be a trend for greater lesion development in caspase-$1^{+/+}$ female mice following oral inoculation with the bacterium as compared with control mice not inoculated with the pathogen, with a 60% increase in atherosclerotic lesion size in these mice, this difference was just short of reaching statistical significance ($p = 0.052$).

5.3.5 *P gingivalis* invasion of the vessel wall

Molecular biology techniques have been used to examine the localization of *P gingivalis* at lesion sites (Haraszthy *et al.*, 2000;Lalla *et al.*, 2003). Although these studies provide clear evidence that *P gingivalis* can gain access to and localize at sites of lesion development, making use of electron microscopy allows us to directly visualize the localization of the bacterium at sites of lesion development.

Following the experimental period, hearts were dissected from one male caspase-$1^{+/+}$ x *apoe*$^{-/-}$ control mouse and one male caspase-$1^{+/+}$ x *apoe*$^{-/-}$ experimental mouse that

had received the oral challenge of *P gingivalis.* Following analysis by electron microscopy of atherosclerotic lesions from these mice, there is evidence that the bacterium is present within the aortic root, at the site of the developing atherosclerotic lesion (**Figure 19**). In these images, there are electron dense bodies in the lesions of mice that were orally challenged with *P gingivlais,* which are indicative of being degraded bacterial products (**Figure 19A**). It is important to note that these electron dense bodies are not seen in the lesions of control mice that were not given the oral challenge of *P gingivalis* (**Figure 19B**). These results lend support to the indication that *P gingivalis* localizes within the vessel wall at the site of lesion development following an oral infection with *P gingivalis.*

Figure 17: Oral infection enhances atherosclerosis in caspase-$1^{+/+}$ male mice.

Mean lesion area of male mice that are either caspase-$1^{+/+}$ *apoe*$^{-/-}$ or caspase-$1^{-/-}$ *apoe*$^{-/-}$ that have been orally challenged with *P gingivalis* (PG) versus those not challenged with the bacterium (control). (**A**) Diagram of the mouse heart showing the region of the ascending aorta where we collect serial sections for the measurement of atherosclerotic lesion size. To determine atherosclerotic size within the ascending aorta, the mean lesion area was be derived from 4 serial Sudan IV stained sections that were cut 10 μm thick, and collected 100 μm apart over a 1 mm segment of the aortic root. (**B**) The mean lesion area was taken as the lesion size for each mouse. Data are shown as means ± SEM. (N= 13 for casp1-/- *apoe*$^{-/-}$, Pg; N= 14 for casp1+/+ *apoe*$^{-/-}$, PG; N= 13 for casp1-/- *apoe*$^{-/-}$, control; N= 13 for casp1+/+ *apoe*$^{-/-}$, control).

#, $p = 0.026$; *, $p = 0.0008$; §, $p = 0.042$

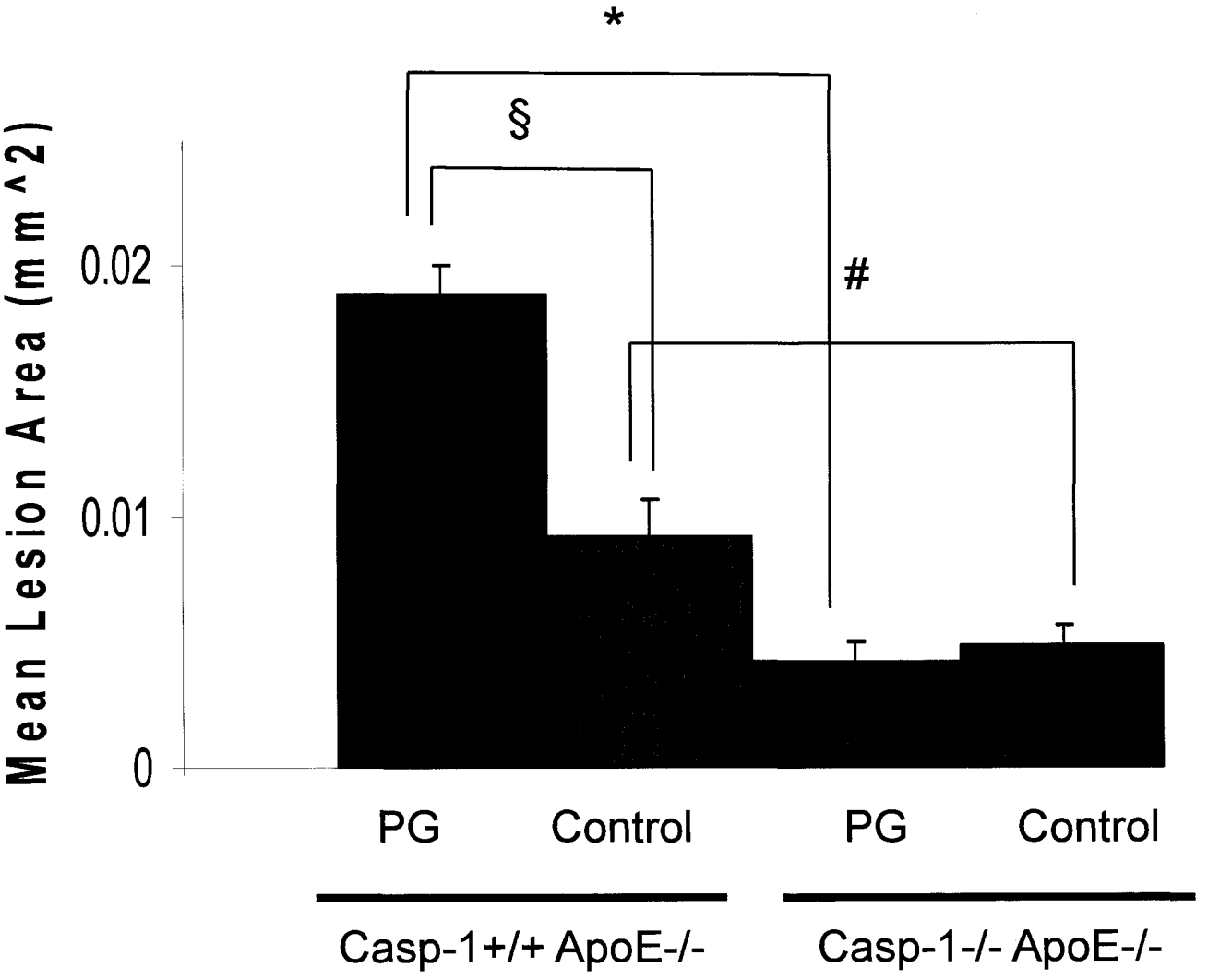

*
§
#
Mean Lesion Area (mm^2)
0.02
0.01
0
PG
Control
PG
Control
Casp-1+/+ ApoE-/-
Casp-1-/- ApoE-/-

Figure 18: Oral infection enhances atherosclerosis in caspase-$1^{+/+}$ female mice.

Mean lesion area of female mice that are either caspase-$1^{+/+}$ *apoe*$^{-/-}$ or caspase-$1^{-/-}$ *apoe*$^{-/-}$ that have been orally challenged with *P gingivalis* (PG) versus those not challenged with the bacterium (control). **(A)** Diagram of the mouse heart showing the region of the ascending aorta where we collect serial sections for the measurement of atherosclerotic lesion size. To determine atherosclerotic size within the ascending aorta, the mean lesion area was be derived from 4 serial Sudan IV stained sections that were cut 10 μm thick, and collected 100 μm apart over a 1 mm segment of the aortic root. **(B)** The mean lesion area was taken as the lesion size for each mouse. Data are shown as means ± SEM. (N= 15 for casp$1^{-/-}$ *apoe*$^{-/-}$, Pg; N= 13 for casp$1^{+/+}$ *apoe*$^{-/-}$, Pg; N= 15 for casp$1^{-/-}$ *apoe*$^{-/-}$, Control; N= 13 for casp$1^{+/+}$ *apoe*$^{-/-}$, Control).

#, $p = 0.043$; *, $p = 0.0009$; §, $p = 0.052$

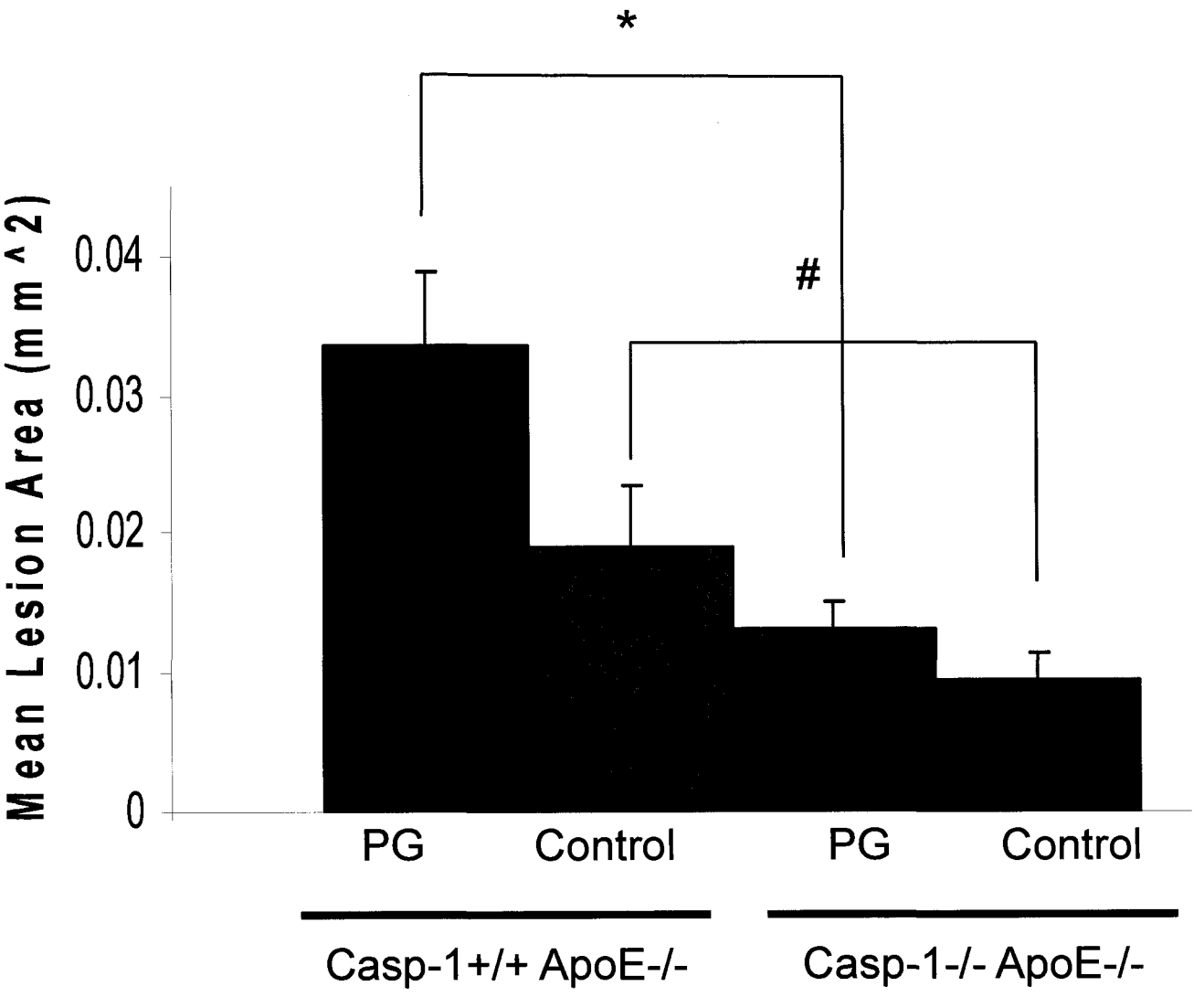
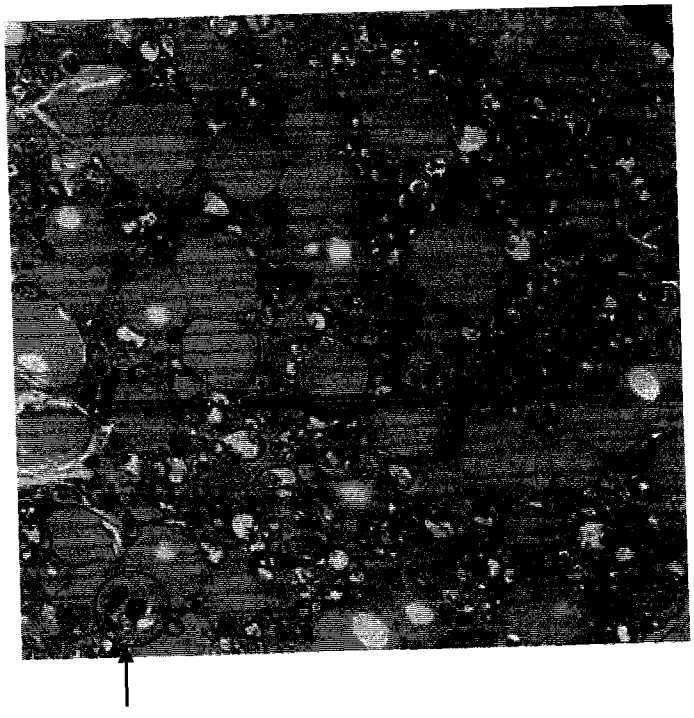

*
#
Mean Lesion Area (m m ^ 2)
0.04
0.03
0.02
0.01
0
PG
Control
PG
Control
Casp-1+/+ ApoE-/-
Casp-1-/- ApoE-/-

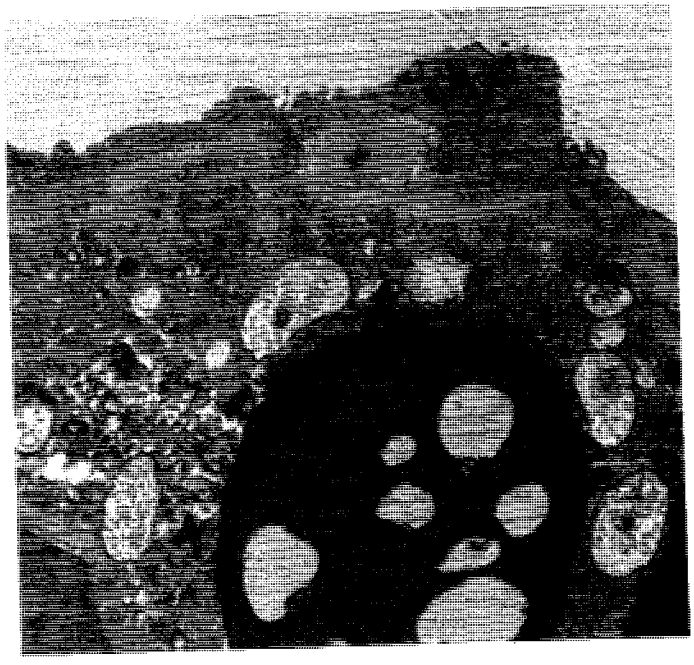
Figure 19: ***P gingivalis*** **localization at lesion sites.**

Hearts were isolated at euthanasia from mice at 17 weeks of age. Lesions formed within the ascending aorta were then examined using electron microscopy. **(A)** Electron microscopy image of an atherosclerotic lesion of a heart isolated from a mouse following oral challenge with *P gingivalis*. Electron dense bodies (indicated by an arrow) are indicative of degraded bacterial products. **(B)** Electron microscopy image of an atherosclerotic lesion of a heart isolated from a control mouse that did not receive the oral challenge with the bacterium.

A.



B.



CHAPTER 6 : DISCUSSION

The relationship between atherosclerosis and periodontal disease was first identified through epidemiological studies. Although a plethora of information exists from both animal studies as well as from human specimens that supports this association, the link between these two diseases remains elusive. In order to understand how these two seemingly distinct diseases are related, it is essential to understand the mechanisms involved. The present study was designed to gain insight into the mechanistic link between periodontal disease and atherosclerosis. Evidence from previous studies suggests that the pro-inflammatory enzyme caspase-1 may play a role in atherosclerotic lesion development but those studies did not provide direct evidence of caspase-1 involvement. Therefore the current study examines the role of caspase-1 in *P gingivalis* mediated atherosclerosis. Employing a mouse model deficient in the caspase-1 enzyme allowed us to test the effect of an oral *Porphyromonas gingivalis* infection in an *in vivo* system.

6.1 Eliminating other risk factors for atherosclerosis

There are a variety of well established risk factors that are known to be associated with the initiation and progression of atherosclerosis. Therefore it was important to establish whether oral infection by *P gingivalis* alters these risk factors for atherosclerosis in the experimental mouse model in order to eliminate these risk factors from the results.

Lalla and collaborators (Lalla *et al.*, 2004) previously examined the effect of *P gingivalis* on the well-known pro-atherogenic factors glucose, insulin, and creatinine *in*

vivo. Based on results from their study, oral inoculation with the *P gingivalis* bacterium does not appear to modulate these established risk factors for atherosclerosis.

Elevated LDL cholesterol levels and obesity are two other risk factors for atherosclerosis (Wilson *et al.*, 1998) that needed to be eliminated as risk factors in our mouse model receiving the oral *P gingivalis* challenge. While it was encouraging that no differences in plasma cholesterol or triglyceride were observed between any of the groups in this study, it was also essential that we examine total lipid levels in the animals. It is possible that even though there is no of difference in total lipid levels, lipid profiles may be altered between groups, manifesting itself, for instance, as a shift towards pro-atherogenic particles, such as VLDL. To eliminate cholesterol levels as a risk factor, we separated plasma lipoproteins by size using FPLC and determined that there were no differences in the distribution of lipoproteins between any groups. In addition to cholesterol levels, body weights of the mice were monitored throughout the experimental period to eliminate obesity as a risk factor. It was determined that no difference in body weights existed in any groups throughout the experimental period. These results are consistent with those seen by Lalla *et al* (Lalla *et al.*, 2004).

Results from the present study indicate that oral inoculation with *P gingivalis* does not modulate cholesterol levels and body weight in the *apoe*$^{-/-}$ mice or the *caspase-1*$^{-/-}$ *apoe*$^{-/-}$ double knockout mice, and thus eliminate both elevated cholesterol levels and obesity as risk factors in our experimental model. Therefore, any observed difference in atherosclerosis in the experimental mouse model can be attributed solely to the participatory role of *P gingivalis* in lesion development.

6.2 Linking periodontal disease and atherosclerosis

Periodontal disease is a chronic infection caused by the bacteria of dental plaque which begins as gingivitis, an inflammation of the soft tissues, and can progress to periodontitis, where destruction of connective tissue and alveolar bone can lead to tooth loss. Among periodontal pathogens, *Porphyromonas gingivalis* has been recognized as a key pathogen and risk factor for periodontal disease (Dzink *et al.*, 1988;Grossi *et al.*, 1994;Loesche *et al.*, 1985). One of the hallmarks of periodontal disease is the complete destruction of the alveolar bone resulting from local infection and inflammation of the periodontal tissues. The amount of alveolar bone loss correlates to the amount of infection in the oral cavity, thus alveolar bone loss can be used as a measurement of the degree of oral infection. In this study there was a significantly greater amount of bone loss in mice orally challenged with *P gingivalis* compared to those not inoculated with the bacterium (Figure 14, Figure 15), indicating that the method of *P gingivalis* challenge in the present study was capable of generating an oral infection.

This study also produced an unexpected result — among control mice that were not given the oral *P gingivalis* challenge, mice competent for the caspase-1 enzyme exhibited greater bone loss than mice deficient in the enzyme (Figure 14, Figure 15). These results were unexpected given that no oral challenge of *P gingivalis* was given to any mice in the control groups. In 2005, Bantel and colleagues (Bantel *et al.*, 2005) examined the involvement of caspase activation in chronic periodontitis with their research being focused on the apoptotic caspases-3 and -9. Isolation of gingival tissue from patients with severe periodontal disease revealed that activation of both caspase-3 and caspase-9 was significantly enhanced in individuals with periodontitis. These data

suggest that the activation of apoptotic caspases is a prominent feature in periodontal tissue destruction and is not dependent on the presence of a *P gingivalis* infection. Although Bantel *et al* (Bantel *et al.*, 2005) focused on apoptotic caspases, the significant decrease in periodontal destruction in the caspase-1 deficient mouse model indicates that the role of caspase-1 in the destruction of alveolar bone warrants further study.

The local infection and inflammation of the periodontal tissues that occurs in periodontal disease results from the host response to the by-products of the bacteria, leading to increased production of pro-inflammatory molecules by the host. A consequence of the host immune response is the loss of epithelial integrity which allows for the bacteria to gain access to the circulation (Lalla *et al.*, 2003). Once in the blood stream, it is possible that the bacterium contributes to lesion development via two separate, yet equally important mechanisms: (1) indirectly through engagement of the host immune response, and (2) directly by localizing at sites of lesion development.

6.2.1 *P gingivalis* promotes atherosclerosis indirectly through engagement of the host immune system

Atherosclerosis is considered to be a modified form of chronic inflammation occurring inside the walls of large and medium sized arteries, involving several components of the immune system, with macrophages playing a central role (Ross, 1999). Given the importance of macrophages in atherogenesis it was of particular importance to examine the relationship between macrophages and *P gingivalis*. Furthermore, it was of particular interest to discover if electron microscopy could be used to capture the interaction between macrophages and *P gingivalis*.

Macrophages are a type of antigen-presenting cell found throughout the body where they act as scavengers, engulfing dead cells and foreign substances along with other debris. They are potent phagocytes that are large enough to engulf invading microbes. Following the engulfing of a foreign microbe by the macrophages, the microbe is degraded into short peptide fragments that are then displayed on the surface of the cell and recognized by the immune system, generating an immune response (Watanabe *et al.*, 1996). In atherosclerosis, macrophages take up modified lipoproteins within the vessel wall to form foam cells, the defining cell of atherosclerosis.

In this study, analysis using electron microscopy (Figure 9) confirmed that macrophages from our mouse model are capable of recognizing and internalizing *P gingivalis in vitro*. These results shed light into how an oral infection with *P gingivalis* could contribute to atherosclerotic lesion development since it is possible that this invasion of macrophages by the bacterium could exacerbate the inflammatory response of atherosclerosis.

It has been suggested that chronic infection with *P gingivalis* may promote atherosclerosis by stimulating the host immune system and altering cytokine expression. Lalla and collaborators (Lalla *et al.*, 2004) originally proposed that *P gingivalis* promotes atherosclerotic lesion development by first gaining access to the oral epithelium, followed by engagement of the host immune response. Bodet and colleagues (Bodet *et al.*, 2006) supported this proposition by providing evidence that when exposed to live *P gingivalis*, pro-inflammatory cytokine expression increases in a human whole blood model *in vitro*. Furthermore, Kesavalu *et al* (Kesavalu *et al.*, 2002) provide evidence that exposure to *P gingivalis in vivo* induced the expression of several pro-inflammatory cytokines.

Zhou *et al* (Zhou *et al.*, 2005) examined the cytokine expression of thioglycollate-elicited peritoneal macrophages exposed to live *P gingivalis* in an attempt to characterize the mechanistic role of the bacterium in the disease process. Results indicated that macrophages sensed *P gingivalis in vitro* which translated into expression of different inflammatory cytokines than macrophages that were not exposed to the bacterium.

While it is interesting to note that macrophages respond to *P gingivalis* by altering their expression of cytokines, no research could be found that examines whether foam cells, which are intricately involved in the development of atherosclerotic lesions, behave the same way as macrophages when exposed to live *P gingivalis*. Foam cells play a pivotal role in the formation of atherosclerotic lesions *in vivo*, however little information is available as to how the immune response to *P gingivalis* exposure differs between foam cells and macrophages. As such, in the present study we were interested in examining whether cytokine expression is altered in macrophages versus foam cells following exposure to the bacterium.

In order to examine how foam cells respond to *P gingivalis* infection, we had to successfully generate foam cells *in vitro*. To ensure that the foam cells we were using for our *in vitro* work would mimic most closely the way foam cells would behave in our *in vivo* mouse model, we decided to generate foam cells from macrophages that were isolated from *apoe*$^{-/-}$ mice since this is our mouse model for atherosclerosis.

Foam cell formation results from the rapid accumulation of intracellular oxLDL within macrophages. By employing a lipid stain (Oil Red O), it is possible to differentiate between a macrophage and a foam cell *in vitro* based on the appearance of

the cell following staining. Since the foam cell is characterized by a cellular accumulation of lipid, they bind more of the lipid stain than their macrophage counterparts. In the current study, by incubating macrophages with modified oxLDL *in vitro* foam cells were generated, as was seen by increased binding of the Oil Red O stain, giving the foam cells a bright red appearance (Figure 11). It is important to note that without this oxLDL treatment macrophages bound minimal amounts of the Oil Red O stain and did not have the same red appearance as those cells incubated with the oxLDL. Since results indicate that our method of forming foam cells *in vitro* was successful, it was now possible to examine the way foam cells behave in comparison with macrophages, in terms of which cytokines that they express, following exposure to live *P gingivalis in vitro.*

Results unique to our study showed that when foam cells were exposed to the live *P gingivalis* bacterium a different cytokine expression profile was generated than when macrophages were exposed to the bacterium (Table 2). Changes in expression of cytokines such as interleukins and adhesion molecules could help to explain how *P gingivalis* is able to promote atherosclerosis *in vivo.*

Interleukins are a group of regulatory proteins that belong to the cytokine family of proteins. They exert their effects *via* stimulation of genes responsible for cellular growth, differentiation, functional cell-surface receptor expression, and cellular effector function (Fisman *et al.*, 2003). Interleukins are produced by a variety of cells, including macrophages, monocytes, neutrophils, and T-cells. In terms of their effect on atherosclerosis as an inflammatory disease, interleukins can be classified into three categories: the noxious interleukins whose activity is considered to be pro-atherogenic;

the protective interleukins whose action has been demonstrated to be athero-protective; and the "neutral" interleukins whose influence on atherosclerosis is indifferent or has not been fully established (Fisman *et al.*, 2003).

Following exposure of foam cells to *P gingivalis*, expression of nearly all of the noxious interleukins increased (these include IL-1α, IL-1β, IL-2, and IL-17) while only one decreased (IL-6) (Table 2). Furthermore, expression of the "neutral" interleukins decreased (IL-3 and IL-5). In addition to changes in the expression of interleukins, expression of cytokines involved in adhesion were increased in foam cells compared to macrophages exposed to *P gingivalis* (Table 2).

Cellular adhesion molecules play critical roles at the interface of a cell and its environment, and have been widely implicated to be involved in the development and/or progression of atherosclerosis (Blankenberg *et al.*, 2003). The adhesion molecule P-selectin is involved in rolling and tethering of endothelial cells and platelets, while vascular cell adhesion molecule-1 (VCAM-1) is involved in the firm adhesion of endothelial cells. Expression of both of these adhesion molecules increased when foam cells were exposed to *P gingivalis* as compared to macrophages (Table 2). Interestingly, there is strong evidence that increased levels of both VCAM-1 and P-selectin are predictors of future cardiovascular disease in humans (Blankenberg *et al.*, 2003). Increased expression of cytokines in foam cells exposed to *P gingivalis* which have been shown to demonstrate pro-atherogenic qualities, such as interleukins and adhesion molecules, outline a possible mechanism by which *P gingivalis* is able to promote atherosclerosis *in vivo*.

While the cytokine profiles of both macrophages and foam cells exposed to live *P gingivalis* generated a large amount of information, one cytokine was of particular interest to the present study – IL-1β. Expression of the pro-inflammatory cytokine IL-1β increased in foam cells generated from *apoe*$^{-/-}$ macrophages following exposure to *P gingivalis* (Table 2). Particular attention was paid to the expression pattern of this cytokine since the caspase-1 enzyme is essential for the maturation of IL-1β. Findings from the current study support results reported by Bodet and colleagues (Bodet *et al.*, 2006), as well as by Zhou *et al* (Zhou *et al.*, 2005), which demonstrate that infection with live *P gingivalis* promotes IL-1β expression in macrophages exposed to the bacterium *in vitro*, and expands on these findings by indicating that IL-1β expression is further increased in foam cells exposed to the bacterium compared with macrophages.

Given that exposure to *P gingivalis* results in increased expression of IL-1β in foam cells, it is logical to presume that there would also be increased expression of the enzyme responsible for the maturation of this cytokine, caspase-1. Even though exposure to the bacterium implied that caspase-1 is activated following infection with *P gingivalis*, these results were suggestive. Consequently we investigated whether *P gingivalis* is capable of activating caspase-1 *in vitro*.

Prior to this study, it was presumed that an increase in IL-1β expression in foam cells following exposure to *P gingivalis* correlated to an increase in caspase-1 expression. In other words, previously there was no direct evidence demonstrating that *P gingivalis* is capable of activating caspase-1. In the present study, we directly examined the relationship between *P gingivalis* infection and the subsequent activation of caspase-1 in mouse peritoneal macrophages. The active site of the caspase-1 enzyme is located on the

p10 subunit therefore the presence of a 10 kDa band is seen as successful activation of the caspase-1 enzyme (Figure 10). The present study provides unique and distinctive results that are extremely encouraging and exciting — we believe that they provide the first direct evidence indicating that *P gingivalis* has the ability to activate caspase-1 *in vitro*. Previous studies have suggested that infection with *P gingivalis* directly activates signal transduction pathways linked to the pro-inflammatory response (Lalla *et al.*, 2003) and here we provide significant evidence that caspase-1 may play a central role as one of the major enzymes essential for the host immune response to *P gingivalis* infection.

6.2.2 *P gingivalis* promotes atherosclerosis directly by localizing at sites of lesion development

Electron microscopy not only allowed us to visualize the interaction between macrophages and *P gingivalis*, but also demonstrated that this procedure can be successful in visualizing *P gingivalis* within these cells. With this in mind, to determine whether the localization of *P gingivalis* within the vasculature at sites of lesion development could be observed electron microscopy was used.

In addition to indirect means by which *P gingivalis* may contribute to atherosclerosis, there is evidence that the bacterium may localize at sites of lesion development once it has gained access to the circulation, thereby directly contributing to the formation of the lesion. Previous studies propose that periodontal pathogens such as *P gingivalis* are capable of adhering to and invading the blood vessel wall at sites of atherosclerotic lesion development, but they do not examine the nature of this localization in that they do not directly examine the vascular wall at lesion sites. In this study, we took the examination of lesion sites one step further by employing electron

microscopy (EM) to directly examine the vasculature for evidence of *P gingivalis* invasion at lesion sites in atherosclerosis susceptible mice.

The notion that chronic infection may exacerbate atherosclerotic lesion development stems from the fact that traditional risk factors for atherosclerosis, such as hypertension, stress, smoking and obesity, do not account for all disease cases. Several putative risk factors for atherosclerosis have been proposed, with periodontal disease emerging as a candidate risk factor following the epidemiological characterization of a link between the severity of periodontal disease and the presence of atherosclerotic lesions in human subjects.

While the means by which periodontal infection influences the initiation and progression of atherosclerotic lesion formation were not well understood prior to our study, it had been suggested that periodontal bacteria contribute to the pathogenesis of atherosclerosis by directly invading the blood vessel wall once it gains access to the circulation (Lalla *et al.*, 2003). Indeed, Dorn and colleagues (Dorn *et al.*, 1999) demonstrated that *P gingivalis* can directly adhere to, invade, and proliferate within human coronary artery smooth muscle cells and human coronary artery endothelial cells *in vitro*. Furthermore, several studies have used molecular biology techniques to demonstrate that periodontal pathogens localize at sites of lesion development *in vivo*. The most compelling evidence prior to our study has come from studies conducted by Haraszthy and colleagues (Haraszthy *et al.*, 2000), as well as by Chiu (Chiu, 1999), indicating that periodontal pathogens, such as *P gingivalis*, are localized within human atheromas using PCR methods. Moreover, Lalla and colleagues (Lalla *et al.*, 2003) isolated DNA from aortic tissue of atherosclerosis susceptible *apoe*$^{-/-}$ mice that had been

orally challenged with *P gingivalis*. The bacterium was once again shown to localize within the aortic tissue of these mice, following analysis by PCR using primers specific for *P gingivalis*. While these studies provide evidence that *P gingivalis* can localize at sites of lesion development *in vivo*, we thought it was of particular importance to directly examine the vascular wall for evidence of *P gingivalis* localization.

In the present study, we used the direct method of electron microscopy (EM) to visually examine the vasculature for evidence of *P gingivalis* invasion at lesion sites in atherosclerosis susceptible mice. Following an oral infection with *P gingivalis*, EM analysis of the ascending aorta revealed evidence of *P gingivalis* localization within the atherosclerotic lesions of these mice (Figure 19). From these results it becomes clear that the bacterium gains access to the vasculature at lesion sites following an oral infection. Prior to this study, evidence was merely suggestive of *P gingivalis* localizing within atherosclerotic lesions, but by employing electron microscopy the present study was able to shift the evidence from suggestive to clearly and directly demonstrating that *P gingivalis* localizes within the blood vessel wall during lesion formation.

Although intact *P gingivalis* bacteria were not observed at lesion sites, degraded bacterial products were identified which were not seen in the control mice. The lack of intact *P gingivalis* bacteria could be explained in part by Dorn *et al* (Dorn *et al.*, 2001) who demonstrated that *P gingivalis* localizes to autophagosomes in human coronary artery endothelial cells *in vitro*. Since autophagy is a degredative pathway, it would be unlikely that live or metabolically active bacteria would persist within the vessel wall for any amount of time.

6.3 *P gingivalis* promotes atherosclerosis *in vivo* via caspase-1

In previous studies (Figure 5) a role for caspase-1 in atherosclerotic lesion development *in vivo* was elucidated by demonstrating that a deficiency in endogenous caspase-1 protected atherosclerosis susceptible male and female *apoe*$^{-/-}$ mice from developing lesions. Additionally, Lalla and colleagues (Lalla *et al.*, 2003) assessed the impact of *P gingivalis* infection on atherogenesis in male *apoE-/-* mice, revealing that oral infection with this periodontal pathogen accelerates early atherosclerosis.

Studies conducted by Chi *et al*, Lalla *et al*, as well as Li *et al* (Chi *et al.*, 2004;Lalla *et al.*, 2003;Li *et al.*, 2002) made use of the *apoe*$^{-/-}$ mouse model to study the involvement of *P gingivalis* in lesion formation, however the attention of these studies was focused only on lesion formation in male mice. Since atherosclerosis is not a gender-specific disease, we sought to examine the role of caspase-1 in lesion formation as a result of *P gingivalis* infection in both male and female atherosclerosis susceptible *apoe*$^{-/-}$ mice.

In the present study, unique results indicating that an oral infection with *P gingivalis* significantly enhances atherosclerotic lesion development in male *apoe*$^{-/-}$ mice that contain a functional caspase-1 enzyme compared to mice not receiving the oral challenge (Figure 17). Furthermore, in male mice deficient in caspase-1 there was no difference in lesion development in those given the oral challenge versus those not challenged with the bacterium. This showed that when we removed caspase-1 from our mouse model, we eliminated the effect that *P gingivalis* had on atherosclerotic lesion

development. These results outline a significant role for caspase-1 in the progression of atherosclerotic lesions in male mice mediated by an oral infection with *P gingivalis*.

While there is a trend for increased atherosclerotic lesion development in female mice competent for the caspase-1 enzyme inoculated with the bacterium (Figure 18), this difference was determined to be just shy of significance ($p = 0.052$). This could explain why previous studies made use of only the male model for atherosclerosis and did not look at lesion development promoted by *P gingivalis* in the female model.

It is well known that there is a gender bias in atherosclerosis, with the disease typically occurring more frequently in the male population than in the female population (Kalin and Zumoff, 1990). While possible causes for the gender difference in the prevalence of atherosclerosis has not been well characterized, researchers have identified sex hormones as the most likely cause of this difference. This dissimilarity in atherogenesis between males and females could help to explain why a difference in atherosclerotic lesion development was observed in male mice following an oral challenge with *P gingivalis* and not in the female mice.

By superimposing the caspase-1 deficiency onto a mouse model already susceptible to atherosclerosis, the current study was able to show the involvement of caspase-1 in *P gingivalis* mediated atherosclerosis (Figure 17, Figure 18). Since the only difference between experimental and control groups is the oral inoculation with *P gingivalis* it is reasonable to conclude that the observed differences are attributable to the bacterial infection.

The present study was set up in an attempt to decipher the mechanisms linking an oral *P gingivalis* infection and atherosclerosis. Here we have identified that caspase-1 is a major enzyme modulating the progression of *P gingivalis* induced atherosclerotic lesion development *in vivo*.

6.4 Caspase-1 and *P gingivalis* in atherosclerosis - Clinical implications

While the present study outlines a possible role for the caspase-1 enzyme in atherosclerosis mediated *P gignivalis* lesion development, the mechanisms by which the bacterium promotes atherosclerosis *in vivo* remain elusive. Still, the identification of caspase-1 as a key enzyme in the disease process associated with *P gingivalis* infection opens the door to examine possible therapeutic targets for the treatment of atherosclerosis in individuals where periodontal disease is the major risk factor.

Colonization of the subgingival tissue by *P gingivalis* resulting in periodontal disease requires several virulence factors including fimbriae, lectin-like adhesins, capsular polysaccharides, lipopolysaccharides, hemagglutinins, hemolysins, and a variety of proteolytic enzymes (Gibson, III and Genco, 2001). Several studies have demonstrated that immunization of animal models with *P gingivalis* antigens confers immunity against *Porphyromonas gingivalis* infections, with most of these studies making use of the various virulence factors possessed by *P gingivalis* in their immunization experiments. In 2001, Frank Gibson and Caroline Genco (Gibson, III and Genco, 2001) examined the ability of immunization with *P gingivalis* specific antibodies to prevent *P gingivalis* associated alveolar bone loss in a mouse model. Mice that were given the immunization were protected against bone loss following infection with

P gingivalis. These results outline a significant possibility that immunization with antibodies specific for *P gingivalis* is a possible treatment for alveolar bone loss associated with periodontal disease. While none of these studies examine the role of immunizations on atherosclerotic lesion formation, given the ability of immunizations to prevent bone loss, it is plausible that immunizations would also delay the development of atherosclerotic lesions where periodontal disease is the major risk factor for disease progression.

While the current study outlines the involvement of caspase-1 in *P gingivalis* mediated atherosclerotic lesion development, the mechanism linking periodontal disease and atherosclerosis is not well-defined, and the role of caspase-1 in other human pathologies remains poorly understood. It is therefore unlikely that caspase-1 would act as a therapeutic target in the treatment of *P gingivalis* associated atherosclerosis until further research can elucidate the mechanisms of caspase-1 in disease processes.

6.5 Limitations of the current study

As discussed by Lalla *et al* (Lalla *et al.*, 2004), the experimental model shared by both of our labs has several advantages over other models of periodontal infection, in that *P gingivalis* is introduced directly into the oral cavity which mimics its natural cycle of infection in humans, rather than the intravenous route employed by other groups. However, given the size of the mouse model, the oral cavity is not large enough to allow for the direct application of the *P gingivalis* bacterium into the sulci, which would most accurately represent the human cycle of periodontal infection. Nevertheless, significant alveolar bone loss (a trademark of periodontal disease) occurred following *P gingivalis*

infection in the mouse model used in the current study, as well as by Lalla *et al* (Lalla *et al.*, 2004), indicating that an oral infection did in fact result following the outlined method of inoculation. Importantly, Lalla *et al* (Lalla *et al.*, 2004) made the observation that periodontal disease does not result from infection by a single microorganism, but rather is the consequence of infection from the variety of microorganisms found within the oral cavity. However, *P gingivalis* has been thoroughly examined with regards to its role in periodontal disease, and has been characterized as one of the most prominent periodontal pathogens in the progression of the disease. Another important point to address is the amount of *P gingivalis* bacterium with which the animals in the present study were inoculated. Although the animals were inoculated with a large amount of *P gingivalis* (10^{10} bacterial cells), Lalla *et al* (Lalla *et al.*, 2004) pointed out that it is likely that only a small fraction of bacteria likely gained access to the oral epithelium and established an oral infection in our experimental model.

CHAPTER 7 : CONCLUSIONS

7.1 Experimental Results

Exposing the underlying mechanisms for atherosclerosis promoted by *P gingivalis* is essential for understanding how two seemingly distinct diseases are related. In the present study we provide a possible mechanism by which an oral infection with *P gingivalis* is able to promote the development of atherosclerotic lesions. By making use of *in vitro* studies, we provide direct evidence that *P gingivalis* is capable of activating the pro-inflammatory enzyme, caspase-1. Moreover, we demonstrated that when foam cells are infected with *P gingivalis*, there is increased expression of the pro-inflammatory cytokine IL-1β. This outlines a possible mechanism by which caspase-1 is involved in *P gingivalis* mediated atherosclerosis. Electron microscopy of atherosclerotic lesions identified *P gingivalis* within lesions, lending support to the notion that *P gingivalis* is capable of gaining access to, and localizing at sites of lesion development. Furthermore, upon analysis of the mandibles of both experimental and control mice, greater bone loss was observed in mice that were challenged with the bacterium indicating that *P gingivalis* is capable of establishing an oral infection based on our method of inoculation. Finally, analysis of the ascending aorta demonstrated increased lesion development in male caspase-$1^{+/+}$ mice that were orally challenged with *P gingivalis*, outlining a role for caspase-1 in the development of *P gingivalis* mediated atherosclerosis. Our findings suggest that oral infection with *P gingivalis* accelerates atherosclerotic lesion development in male *apoe*$^{-/-}$ mice, at least in part through activation of the caspase-1 enzyme.

7.2 Proposed mechanism of *P gingivalis* mediated atherosclerosis

The role of inflammatory caspases in human pathologies has not been well studied and remains virtually unknown. Uncovering the mechanisms by which caspase-1 promotes early atherosclerotic lesion development following an oral infection with *P gingivalis* is critical for understanding the link between oral infection and atherosclerosis. In the current study, an oral infection with *P gingivalis* leads to periodontal disease which is seen in the form of alveolar bone destruction. The bacteria gain access to the circulation, possibly by invading the well-vascularized periodontal connective tissues (Bodet *et al.*, 2006). Once released into the circulation, *P gingivalis* gains access to the vasculature by directly invading the vessel wall at sites of lesion development. Once the bacterium has gained access to, and localized at sites of lesion development, they may directly activate the caspase-1 signalling cascade, resulting in an exacerbated immune response in the form of increased expression of the pro-inflammatory cytokines, IL-18 and IL-1β.

CHAPTER 8 : FUTURE DIRECTIONS

In the current study we addressed the role of caspase-1 in the progression of atherosclerotic lesion development following an oral challenge with *P gingivalis*. This study was essential in allowing us to examine the relationship between caspase-1 activation and atherosclerosis following *P gingivalis* infection, and has provided insight into the mechanism by which oral infection with *P gingivalis* is able to promote lesion development. With that being said, a detailed mechanism of the individual steps involved in the progression from oral infection to atherosclerosis still remains elusive. To continue our work, we propose a series of *in vivo* mouse atherosclerosis experiments that will define the signalling pathways that lead to *P gingivalis* induced atherosclerotic lesion development.

8.1.1 Inflammasome complexes and *P gingivalis* mediated atherosclerosis

Although a detailed mechanism of the steps required for caspase-1 activation have yet to be elucidated, it has been demonstrated that activation of the caspase-1 enzyme requires the proper formation of a molecular platform termed the inflammasome complex (Lamkanfi *et al.*, 2007;Mariathasan *et al.*, 2004;Martinon and Tschopp, 2004). The adaptor proteins, ASC and Ipaf, are essential for the proper formation of two distinct inflammasome complexes (Lamkanfi *et al.*, 2007;Mariathasan *et al.*, 2004). What remains unknown is which of these inflamamsome complexes are responsible for the activation of caspase-1 and the subsequent acceleration of early atherosclerotic lesion formation. Studies outlined by our lab have been designed to compare lesion development in two groups of atherosclerosis susceptible *apoe*$^{-/-}$ mice that have been

orally challenged with the *P gingivalis* bacterium: (1) those mice competent or deficient for the ASC adaptor protein, and (2) those mice competent or deficient for the Ipaf adaptor protein. Results from this study will allow us to define which inflammasome complex promotes atherosclerosis by initiating caspase-1 activation following an oral infection with *P gingivalis.*

8.1.2 A role for IL-18 and/or IL-1β in *P gingivalis* mediated lesion development

Furthermore, caspase-1 has been identified as the enzyme responsible for the maturation of two pro-inflammatory cytokines, IL-18 and IL-1β (Gu *et al.*, 1997;Kuida *et al.*, 1995;Loppnow *et al.*, 1998;Martinon and Tschopp, 2004). What remains unknown is whether the ability of caspase-1 to promote atherosclerosis in mice given an oral challenge with *P gingivalis* is linked to its ability to induce the expression of IL-18 or IL-1β. By superimposing a deficiency in IL-18 or IL-1β onto *apoe*$^{-/-}$ mice that are susceptible to atherosclerosis, we will be able to identify which pro-inflammatory cytokine(s) is involved in the progression of atherosclerosis following *P gingivalis* infection.

8.1.3 The role of TLR-2 and TLR-4 in atherosclerosis promoted by *P gingivalis*

Finally, there is evidence that both TLR2 and TLR4 sense and respond to different components of the *P gingivalis* bacterium (Burns *et al.*, 2006;Coats *et al.*, 2003), and that *P gingivalis* activates the TLR signalling pathway resulting in the production of several pro-inflammatory cytokines, including the caspase-1 dependent IL-1β (Hajishengallis *et al.*, 2004). Studies designed by our lab propose to make use of *apoe*$^{-/-}$ mice deficient in either TLR-2 or TLR-4. Results from this proposed study will aid in defining the role of TLR-2 and TLR-4 in *P gingivalis* induced atherosclerosis.

REFERENCES

Akira S, Takeda K (2004). Toll-like receptor signalling. *Nat Rev Immunol* 4(7):499-511.

Al-Zahrani MS, Bissada NF, Borawskit EA (2003). Obesity and periodontal disease in young, middle-aged, and older adults. *J Periodontol* 74(5):610-615.

Andrian E, Grenier D, Rouabhia M (2004). In vitro models of tissue penetration and destruction by Porphyromonas gingivalis. *Infect Immun* 72(8):4689-4698.

Bantel H, Beikler T, Flemmig TF, Schulze-Osthoff K (2005). Caspase activation is involved in chronic periodontitis. *FEBS Lett* 579(25):5559-5564.

Blankenberg S, Barbaux S, Tiret L (2003). Adhesion molecules and atherosclerosis. *Atherosclerosis* 170(2):191-203.

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM *et al.* (2003). A unified model for apical caspase activation. *Mol Cell* 11(2):529-541.

Bodet C, Chandad F, Grenier D (2006). Porphyromonas gingivalis-induced inflammatory mediator profile in an ex vivo human whole blood model. *Clin Exp Immunol* 143(1):50-57.

Burns E, Bachrach G, Shapira L, Nussbaum G (2006). Cutting edge: TLR2 is required for the innate response to *Porphyromonas gingivalis*: activation leads to bacterial persistence and TLR2 deficiency attenuates induced alveolar bone loss resorption. *The Journal of Immunology* 177:8296-8300.

Chi H, Messas E, Levine RA, Graves DT, Amar S (2004). Interleukin-1 receptor signaling mediates atherosclerosis associated with bacterial exposure and/or a high-fat diet in a murine apolipoprotein E heterozygote model: pharmacotherapeutic implications. *Circulation* 110(12):1678-1685.

Chiu B (1999). Multiple infections in carotid atherosclerotic plaques. *Am Heart J* 138(5 Pt 2):S534-S536.

Coats SR, Reife RA, Bainbridge BW, Pham TT, Darveau RP (2003). Porphyromonas gingivalis lipopolysaccharide antagonizes Escherichia coli lipopolysaccharide at toll-like receptor 4 in human endothelial cells. *Infect Immun* 71(12):6799-6807.

Cooper AM, Magram J, Ferrante J, Orme IM (1997). Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *J Exp Med* 186(1):39-45.

Creagh EM, Conroy H, Martin SJ (2003). Caspase-activation pathways in apoptosis and immunity. *Immunol Rev* 193:10-21.

Decken K, Kohler G, Palmer-Lehmann K, Wunderlin A, Mattner F, Magram J *et al.* (1998). Interleukin-12 is essential for a protective Th1 response in mice infected with Cryptococcus neoformans. *Infect Immun* 66(10):4994-5000.

Deshpande RG, Khan MB, Genco CA (1998). Invasion of aortic and heart endothelial cells by Porphyromonas gingivalis. *Infect Immun* 66(11):5337-5343.

Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR, Jr., Papapanou PN *et al.* (2003). Relationship between periodontal disease, tooth loss, and carotid artery plaque: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). *Stroke* 34(9):2120-2125.

Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR, Jr., Sacco RL *et al.* (2005). Periodontal microbiota and carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). *Circulation* 111(5):576-582.

Dorn BR, Burks JN, Seifert KN, Progulske-Fox A (2000). Invasion of endothelial and epithelial cells by strains of Porphyromonas gingivalis. *FEMS Microbiol Lett* 187(2):139-144.

Dorn BR, Dunn WA, Jr., Progulske-Fox A (2001). Porphyromonas gingivalis traffics to autophagosomes in human coronary artery endothelial cells. *Infect Immun* 69(9):5698-5708.

Dorn BR, Dunn WA, Jr., Progulske-Fox A (1999). Invasion of human coronary artery cells by periodontal pathogens. *Infect Immun* 67(11):5792-5798.

Duncan M, Dewhirst F, Chen T (2002). The *Porphyromonas gingivalis* genome project. The Forsyth Institute; The Institute for Genomic Research.

Dzink JL, Socransky SS, Haffajee AD (1988). The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol* 15(5):316-323.

Fisman EZ, Motro M, Tenenbaum A (2003). Cardiovascular diabetology in the core of a novel interleukins classification: the bad, the good and the aloof. *Cardiovasc Diabetol* 2:11.

Gibson FC, III, Genco CA (2001). Prevention of Porphyromonas gingivalis-induced oral bone loss following immunization with gingipain R1. *Infect Immun* 69(12):7959-7963.

Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE *et al.* (1994). Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* 65(3):260-267.

Gu Y, Kuida K, Tsutsui H, Ku G, Hsiao K, Fleming MA *et al.* (1997). Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. *Science* 275(5297):206-209.

Hajishengallis G, Sojar H, Genco RJ, DeNardin E (2004). Intracellular signaling and cytokine induction upon interactions of Porphyromonas gingivalis fimbriae with pattern-recognition receptors. *Immunol Invest* 33(2):157-172.

Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ (2000). Identification of periodontal pathogens in atheromatous plaques. *J Periodontol* 71(10):1554-1560.

Hashimoto T, Kihara M, Yokoyama K, Fujita T, Kobayashi S, Matsushita K *et al.* (2003). Lipoxygenase products regulate nitric oxide and inducible nitric oxide synthase production in interleukin-1beta stimulated vascular smooth muscle cells. *Hypertens Res* 26(2):177-184.

Haynes WG, Stanford C (2003). Periodontal disease and atherosclerosis: from dental to arterial plaque. *Arterioscler Thromb Vasc Biol* 23(8):1309-1311.

Kalin MF, Zumoff B (1990). Sex hormones and coronary disease: a review of the clinical studies. *Steroids* 55(8):330-352.

Kawakami K, Koguchi Y, Qureshi MH, Miyazato A, Yara S, Kinjo Y *et al.* (2000). IL-18 contributes to host resistance against infection with Cryptococcus neoformans in mice with defective IL-12 synthesis through induction of IFN-gamma production by NK cells. *J Immunol* 165(2):941-947.

Kesavalu L, Chandrasekar B, Ebersole JL (2002). In vivo induction of proinflammatory cytokines in mouse tissue by Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans. *Oral Microbiol Immunol* 17(3):177-180.

Kirii H, Niwa T, Yamada Y, Wada H, Saito K, Iwakura Y *et al.* (2003). Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 23(4):656-660.

Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS *et al.* (1995). Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* 267(5206):2000-2003.

Lalla E, Lamster IB, Feit M, Huang L, Schmidt AM (1998). A murine model of accelerated periodontal disease in diabetes. *J Periodontal Res* 33(7):387-399.

Lalla E, Lamster IB, Hofmann MA, Bucciarelli L, Jerud AP, Tucker S *et al.* (2003). Oral infection with a periodontal pathogen accelerates early atherosclerosis in apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol* 23(8):1405-1411.

Lalla E, Park DB, Papapanou PN, Lamster IB (2004). Oral disease burden in Northern Manhattan patients with diabetes mellitus. *Am J Public Health* 94(5):755-758.

Lamkanfi M, Kanneganti TD, Franchi L, Nunez G (2007). Caspase-1 inflammasomes in infection and inflammation. *J Leukoc Biol* 82(2):220-225.

Lamont RJ, Jenkinson HF (1998). Life below the gum line: pathogenic mechanisms of Porphyromonas gingivalis. *Microbiol Mol Biol Rev* 62(4):1244-1263.

Lee JK, Kim SH, Lewis EC, Azam T, Reznikov LL, Dinarello CA (2004). Differences in signaling pathways by IL-1beta and IL-18. *Proc Natl Acad Sci U S A* 101(23):8815-8820.

Li L, Messas E, Batista EL, Jr., Levine RA, Amar S (2002). Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. *Circulation* 105(7):861-867.

Loesche WJ, Syed SA, Schmidt E, Morrison EC (1985). Bacterial profiles of subgingival plaques in periodontitis. *J Periodontol* 56(8):447-456.

Loppnow H, Werdan K, Reuter G, Flad HD (1998). The interleukin-1 and interleukin-1 converting enzyme families in the cardiovascular system. *Eur Cytokine Netw* 9(4):675-680.

Lucas AD, Greaves DR (2001). Atherosclerosis: role of chemokines and macrophages. *Expert Rev Mol Med* 2001:1-18.

Lusis AJ (2000). Atherosclerosis. *Nature* 407(6801):233-241.

Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP *et al.* (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430(6996):213-218.

Marsh P (1989). Host defences and microbial homeostasis: role of microbial interactions. *J Dent Res* 68:1567-1575.

Martinon F, Tschopp J (2004). Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 117(5):561-574.

Molloy J, Wolff LF, Lopez-Guzman A, Hodges JS (2004). The association of periodontal disease parameters with systemic medical conditions and tobacco use. *J Clin Periodontol* 31(8):625-632.

Munder M, Mallo M, Eichmann K, Modolell M (1998). Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. *J Exp Med* 187(12):2103-2108.

Nassar H, Chou HH, Khlgatian M, Gibson FC, III, Van Dyke TE, Genco CA (2002). Role for fimbriae and lysine-specific cysteine proteinase gingipain K in expression of interleukin-8 and monocyte chemoattractant protein in Porphyromonas gingivalis-infected endothelial cells. *Infect Immun* 70(1):268-276.

Nicholson DW (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* 6(11):1028-1042.

Papapanou PN (1999). Epidemiology of periodontal diseases: an update. *J Int Acad Periodontol* 1(4):110-116.

Pascual RM, Awsare BK, Farber SA, Panettieri RA, Jr., Peters SP, Penn RB (2003). Regulation of phospholipase A2 by interleulin-1 in human airway smooth muscle. *Chest* 123(3 Suppl):433S-434S.

Petrilli V, Papin S, Tschopp J (2005). The inflammasome. *Curr Biol* 15(15):R581.

Plump AS, Breslow JL (1995). Apolipoprotein E and the apolipoprotein E-deficient mouse. *Annu Rev Nutr* 15:495-518.

Poyet JL, Srinivasula SM, Tnani M, Razmara M, Fernandes-Alnemri T, Alnemri ES (2001). Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J Biol Chem* 276(30):28309-28313.

Quinn MT, Parthasarathy S, Fong LG, Steinberg D (1987). Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci U S A* 84(9):2995-2998.

Ramage P, Cheneval D, Chvei M, Graff P, Hemmig R, Heng R *et al.* (1995). Expression, refolding, and autocatalytic proteolytic processing of the interleukin-1 beta-converting enzyme precursor. *J Biol Chem* 270(16):9378-9383.

Rautemaa R, Jarvensivu A, Kari K, Wahlgren J, DeCarlo A, Richardson M *et al.* (2004). Intracellular localization of Porphyromonas gingivalis thiol proteinase in periodontal tissues of chronic periodontitis patients. *Oral Dis* 10(5):298-305.

Ross R (1999). Atherosclerosis--an inflammatory disease. *N Engl J Med* 340(2):115-126.

Shi Y (2002). Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* 9(3):459-470.

Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W, Jr., Rosenfeld ME *et al.* (1994). A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb* 14(5):840-856.

Steinberg D, Witztum JL (1990). Lipoproteins and atherogenesis. Current concepts. *JAMA* 264(23):3047-3052.

Tortora G, Grabowski S (2003). Principles of anatomy and physiology. 10 ed. John Wiley & Sons, Inc.

Trinchieri G, Sher A (2007). Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 7(3):179-190.

Vainio S, Ikonen E (2003). Macrophage cholesterol transport: a critical player in foam cell formation. *Ann Med* 35(3):146-155.

Vance DE, Vance JE (2002). Biochemistry of lipids, lipoproteins, and membranes. 4 ed. Elsevier.

Wang PL, Ohura K (2002). Porphyromonas gingivalis lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. *Crit Rev Oral Biol Med* 13(2):132-142.

Watanabe T, Haraoka S, Shimokama T (1996). Inflammatory and immunological nature of atherosclerosis. *Int J Cardiol* 54 Suppl:S51-S60.

Whitman SC (2004). A practical approach to using mice in atherosclerosis research. *Clin Biochem Rev* 25:81-93.

Whitman SC, Rateri DL, Szilvassy SJ, Yokoyama W, Daugherty A (2004). Depletion of natural killer cell function decreases atherosclerosis in low-density lipoprotein receptor null mice. *Arterioscler Thromb Vasc Biol* 24(6):1049-1054.

Whitman SC, Ravisankar P, Daugherty A (2002a). Interleukin-18 enhances atherosclerosis in apolipoprotein E(-/-) mice through release of interferon-gamma. *Circ Res* 90(2):E34-E38.

Whitman SC, Ravisankar P, Daugherty A (2002b). IFN-gamma deficiency exerts gender-specific effects on atherogenesis in apolipoprotein E-/- mice. *J Interferon Cytokine Res* 22(6):661-670.

Whitman SC, Ravisankar P, Elam H, Daugherty A (2000). Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice. *Am J Pathol* 157(6):1819-1824.

Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB (1998). Prediction of coronary heart disease using risk factor categories. *Circulation* 97(18):1837-1847.

Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* 75(4):641-652.

Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F *et al.* (2004). Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* 364(9438):937-952.

Zhou Q, Desta T, Fenton M, Graves DT, Amar S (2005). Cytokine profiling of macrophages exposed to Porphyromonas gingivalis, its lipopolysaccharide, or its FimA protein. *Infect Immun* 73(2):935-943.