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Effect of whole smaoke on secretion of IL-1beta, TNF-alpha, TGF-beta and $PGE_2$ by peripheral blood mononuclear cells



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# Effect of Whole Smoke on Secretion of IL-1β, TNF-α, TGF-β and $PGE_2$ by Peripheral Blood Mononuclear Cells

by

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THESIS

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**Effect of Whole Smoke on Secretion of IL-1β, TNF-α, TGF-β and $PGE_2$ by Peripheral Blood Mononuclear Cells. Mahasti Saghizadeh, D.D.S.**

Alterations of the host response caused by short-term exposure to high levels of smoke during the act of smoking (acute smoke exposure) as well as long-term exposure to lower levels of tobacco substances in the bloodstream of smokers (chronic smoke exposure) may play a role in the pathogenesis of periodontal diseases in smokers. In this study, we examined the secretion of four cytokines [interleukin (IL)-1β, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and prostaglandin $E_2$ ($PGE_2$) ] from mononuclear blood cells from current smokers and nonsmokers exposed to in vitro tobacco smoke (which may be comparable to in vivo acute smoke exposure) and mononuclear blood cells from current smokers not exposed to further in vitro smoke (which may be comparable to chronic smoke exposure). Peripheral blood mononuclear cells were isolated from eight healthy current smokers and eight healthy nonsmokers, plated in culture wells, exposed in vitro for 1–5 minutes to cigarette smoke in a smoke box system or not exposed (baseline controls), and then incubated without further smoke exposure for another 24 hours. Supernatants from each well were then collected and assayed for the concentrations of the four cytokines by enzyme-linked immunosorbent assay (ELISA). At baseline, mean IL-1β levels were higher in smokers than in nonsmokers (mean: 10.6 vs. 5.9 pg/ml, anova: $P < 0.05$). In both smokers and nonsmokers, secreted levels of IL-1β increased from 0 to 5 minutes of in vitro smoke exposure (mean: 5.9–9.9 pg/ml, t-test: $P < 0.05$ for nonsmokers only) with levels in smokers higher than in nonsmokers ($P > 0.05$). Mean TNF-α levels increased from 0 to 2 minutes of smoke exposure and decreased from 2 to 5 min in smokers and nonsmokers, with higher levels in nonsmokers than smokers at all time points ($P > 0.05$). Mean TGF-β levels were higher in smokers than in nonsmokers at all time points (mean: 180.5 vs. 132.0 pg/ml, $P < 0.05$ at 5 minutes only) with no significant alteration of the pattern of secretion with cigarette smoke exposure. There was no significant difference between $PGE_2$ in nonsmokers and smokers at baseline.

There was an increase in $PGE_2$ secretion from baseline to 5 minutes in both smokers and nonsmokers with no significant difference between two groups. These observed alterations in the secretion of cytokines from mononuclear blood cells in smokers, relative to nonsmokers, and with in vitro smoke exposure may play a role in the pathogenesis of periodontal diseases in smokers.

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## Introduction

Tobacco use in general, and cigarette smoking in particular, is a major risk factor for periodontal diseases.[1] Cigarette smoking is associated with increased alveolar bone loss[2], and poor response to periodontal therapy.[3,4] Cigarette smoking is known to affect the systemic and local immune responses.[5-8] Loss of alveolar bone and connective tissue attachment in periodontitis results from interactions between certain bacteria colonizing tooth surfaces and the host response. It is likely that smoke influences these host-bacterial interactions.

Alterations of the host response caused by short-term exposure to high levels of smoke during the act of smoking (acute smoke exposure) as well as long-term exposure to lower levels of tobacco substances in the blood stream of smokers (chronic smoke exposure) may play a role in the pathogenesis of periodontal diseases in smokers. In this study, we examined the secretion of four cytokines from mononuclear blood cells from current smokers and nonsmokers exposed to *in vitro* tobacco smoke and mononuclear blood cells from current smokers not exposed to further *in vitro* smoke. The studied cytokines were interleukin-1β, tumor necrosis factor-α, transforming growth factor-β and prostaglandin $E_2$.

An overview and some background literatures are provided for the related subjects.

### Smoking

It has been demonstrated that smoking is a risk factor for periodontitis in adults. The number of pack/years of exposure to tobacco smoke is associated with increased risk for chronic periodontitis and increased disease severity in smokers compared to nonsmokers.[9,10] Smoking

has also been shown to be associated with increased disease severity in aggressive periodontitis.[11] The pathologic mechanisms proposed for the detrimental effects of smoking on the periodontium include alterations of the periodontal tissue vasculature, direct altering effects on the bacterial microflora, and on various components of the host response in periodontal diseases.[12] One way of understanding the host response in periodontal diseases is through the Critical Path Model.[13] In this model, the host first attempts to neutralize the periodontal pathogens in the pocket through various defense mechanisms such as complement, antibodies, and subsequent neutrophil clearance. After these defenses are exhausted, the bacteria and bacterial products may penetrate through the sulcular and junctional epithelium into the underlying periodontal connective tissue. There, monocytes and lymphocytes along with other cells of the periodontium may elicit an inflammatory response through several inflammatory cytokines and chemokines such as interleukin-1β (IL-1β), Tumor Necrosis Factor (TNF-α), and prostaglandins such as $PGE_2$.[12,13] These substances have multiple and overlapping effects on the periodontium such as increased phagocytic activity and osteoclastic bone resorption, release of proteolytic enzymes, and recruitment and activation of other components of the host response. While some of these effects may be beneficial in neutralizing periodontal bacteria, the overall effect of this inflammatory response is periodontal destruction. In the absence of bacterial components and products, monocytes, lymphocytes, and other cells may secrete a second class of mediators that may facilitate maintenance, repair, and regeneration of lost periodontal support.[13] These mediators, or "reparative cytokines", include a variety of growth factors such as Transforming Growth Factor (TGF-β).

Cigarette smoking affects the immune and inflammatory system in many different ways. It reduces antibody production,[14] inhibits several peripheral blood neutrophil functions,[4,15-17] and

chemotactic and phagocytotic activities. [18,19] It has been shown that exposure to tobacco smoke is associated with a decreased percent and activity of NK cells in humans and animals. [20,21] This could result in decreased defense against pathogens. Miller et al. (1982) showed that the percent of total T lymphocyte as well as the CD8 subset were increased, whereas the percent of CD4 cells decreased in heavy smokers. [22] Quinn et al. (1996) showed that smoking could suppress the production of $IgG_2$ in generalized aggressive periodontitis. [23] Since $IgG_2$ is mainly regulated by macrophages[24] and smoking has been shown to have effects on macrophages[25] and on T-lymphocyte subsets ratios,[26] smoking may compromise antibody production through modulating macrophage and T-helper cell functions.

Serum IgE levels have been shown to be increased in adults[27] and experimental animals[28] exposed to tobacco smoke. This possibly could lead to more tissue destruction in the presence of pathogens. Byrd et al. (1994) found that phytohaemagglutinin (PHA)-induced IL-4 production by peripheral blood mononuclear cells of smokers is significantly higher than that of non-smokers; and heavy smokers produce more IL-4 than light smokers. [29] It was suggested that an imbalance in cytokine production might be partly responsible for the increase in serum IgE.

Acute and chronic exposure to tobacco smoke has additional local effects on tissue. Alveolar macrophages from smokers exhibit decreased antibody-dependent cell-mediated cytotoxicity.[30] Induced sputum from smokers was found to have a higher percent of macrophages and a lower proportion of neutrophils. The percent of macrophages expressing surface molecules associated with antigen-presenting functions (e.g., HLA-DR, CD54) was also found to be significantly lower in smokers than in nonsmokers.[31]

It has been shown that smoking causes a reduction in the gingival blood flow with a decreased number of circulating cells and less oxygen reaching the gingiva, thus weakening its

defense and reparative ability. The mechanism by which smoking can cause vasoconstriction is not completely understood but it is most likely mediated through the nervous system. It has also been shown that smoking reduces the functional activity of leukocytes and macrophages in saliva and crevicular fluid, as well as decreasing the chemotaxis and phagocytosis of blood and tissue polymorphonuclear neutrophils (PMNs).[16,17,32,33]

Other studies have shown that tobacco smoking is associated with a reduction in the short-term oxidation-reduction potentials in dental plaque and oxygen levels, thereby resulting in an increased proportion of anaerobic bacteria[34] and decreased PMN mobility [35] thus contributing to increased susceptibility to bacterial infection.

The negative immunological effects of nicotine are believed to involve several possible mechanisms: 1. Hypersecretion of glucocorticoid hormones[36-39] ; 2. Direct effect on lymphocytes through activation of nicotinic cholinergic receptors[40] ; 3. Stimulation of a wide array of pituitary hormones and /or sympathetic nervous system , including the release of peripheral catecholamines[38], which will cause disturbances in immune responsiveness; 4. Serving as a hapten [41] ; 5. Possible activation of central nicotinic-cholinergic receptors.[42]

**Monocytes**

The mononuclear phagocytes (monocytes/macrophages) are major producers of inflammatory mediators. Monocytes form 2-3 % of total white blood cells. They produce a wide variety of inflammatory mediators.

Factors that affect secretion capacity of monocytes include: genetic, pathogens, and behavioral and environmental factors such as dietary fat intake, stress and tobacco. A hyperinflammatory phenotype is seen in some conditions such as type 1 diabetes[43] and aggressive

periodontitis.[44] A variety of components of pathogens are shown to affect the secretion abilities of monocytes, but the focus of most studies is endotoxin (lipopolysaccharide, LPS).[45,46] It has been shown that fat intake may enhance monocyte secretion of inflammatory cytokines.[47,48] There are two principal immunological responses to stress one leads to neutrophil impairment and the other leads to monocytic upregulation.[49,50]

Two outcomes of hypersensitive monocytes are periodontitis and coronary heart disease. It has been speculated in recent years that periodontitis may contribute to the development of coronary heart disease, which is a multifactorial disease.[51] Periodontitis and atherosclerosis have many potential pathogenic mechanisms in common. Both diseases have genetic and gender predisposition, and potentially share many risk factors, the most significant of which may be smoking status. [52] Independent risk factors for atherosclerosis and its consequences include age, male gender, smoking, hypercholesterolemia, systemic hypertension, plasma fibrinogen, white cell count, hematocrit and diabetes mellitus[53,54] ; all of which are also associated with periodontal disease, with the exception of hypercholesterolemia and systemic hypertension.[53,55]

Studies have demonstrated hyper-responsiveness of monocytes from aggressive periodontitis patients with respect to their production of $PGE_2$ in response to LPS[43,56] , and in refractory periodontitis patients. [57,58] This hyper-responsive phenotype could lead to increased connective tissue or bone loss due to inappropriately excessive production of these catabolic factors.

There is increasing evidence that tissue destruction in periodontitis lesions is mostly a result of mobilization of the host defenses via activation of monocytes, lymphocytes, fibroblasts, and other host cells. Bacterial factors, in particular bacterial LPS, are thought to stimulate production of cytokines and inflammatory mediators including arachidonic acid metabolites such

as prostaglandin $E_2$ ($PGE_2$). Such cytokines and inflammatory mediators in turn promote the release of tissue-derived enzymes, the matrix metalloproteinases, which are destructive to the extracellular matrix and bone. [13,59] Even though monocytes/macrophages represent only 3.5% of total cell counts in progressing periodontitis sites, these cells are capable of producing large amounts of $PGE_2$ and interleukin-1β. [60,61]

## Cytokines

Cytokines are molecules released by host cells into the local environment; provide molecular signals to other cells thereby affecting their function. Among the cytokines and inflammatory mediators found to be associated with periodontitis are: interleukin-1β, prostaglandin $E_2$, tumor necrosis factor-α, and transforming growth factor-β.

### *Interleukin 1-beta (IL-1β)*

IL-1β is a significant proinflammatory cytokine. Human IL-1β is synthesized as a 269 amino acid, 31-kDa-precursor protein (prepro-IL-1β) that is cleaved by IL-1β-converting enzyme (ICE) to the 153 amino acid, 17 kDa mature IL-1β plus a prosegment. [62,63] A combination of the mature form, the prosegment and prepro-IL-1β is released from the cell.

IL-1β is produced primarily by monocytes and macrophages[64] but also by astrocytes, oligodendroglia, adrenal cortical cells, natural killer (NK cells), endothelial cells, keratinocytes, platelets, neutrophils, osteoblasts, T cells, fibroblasts. [65-67] The most extensively studied function of IL-1β is initiation of inflammation. Bacterial endotoxin or a variety of non-microbial inflammatory substances induce production of IL-1, which is released into the local environment.

It has been shown that during experimental gingivitis, IL-1β increases in gingival crevicular fluid during plaque formation.[68] IL-1β has been implicated in progressive periodontitis and stimulation of bone resorption.[69] Studies suggest a genetic influence on levels of interleukin-1β in gingival fluids.[70-72] Some studies have found that polymorphisms in genes of the IL-1 family are associated with a higher severity of periodontitis [73-75], while others found no association.[76,77] It is hypothesized that IL-1β is likely a contributing factor to the more advanced periodontitis seen in users of tobacco products. It has also been shown that there is a gene-environment interaction between smoking and the IL-1 genetic polymorphism. Smokers bearing the genotype positive IL-1 allele combination have an increased risk of periodontitis.[78]

***Tumor Necrosis Factor Alpha (TNF-α)***

TNF-α is a significant proinflammatory cytokine. TNF-α is produced by monocytes, neutrophils, activated T and B-lymphocytes, NK cells, astrocytes, endothelial cells, smooth muscle cells, and some transformed cells.[79,80] Mature human TNF-α is a polypeptide of 157 amino acids residues.[79] The biologically active native form of TNF-α is a trimer.[81,82]

TNF-α is initially synthesized as a larger protein with the mature 17-kDa factors comprising the C-terminal portion of this precursor. The N-terminal sequence of the precursor contains both hydrophilic and hydrophobic domains and its presence results in the occurrence of TNF-α as a membrane-bound form from which the mature factor is released by proteolytic cleavage.[83-85] Evidence suggests that the membrane-anchored form of TNF-α on the surface of macrophages and/or monocytes, in addition to serving as a reservoir for release of soluble TNF-α has lytic activity and may also have an important role in intercellular communication.[83-85]

TNF-α is secreted predominantly by monocytes/macrophages during inflammation. It induces IL-1 release by macrophages in experimental animals.[86]

### *IL-1β and TNF-α*

IL-1 and TNF are important mediators of inflammatory responses and appear to play a central role in the pathogenesis of many chronic inflammatory diseases.[87,88] It is now well documented that their biological activities in vivo is sufficient to reproduce local inflammation and matrix catabolism[89] by attracting and activating white blood cells to tissues and stimulating their secretion of other lymphocytotropic cytokines and catabolic enzymes. Higher production of these cytokines has also been associated with response to infection, where local production of IL-1 and TNF-α facilitates the elimination of the microbial invasion. Classic studies, however, also report that in some infectious conditions, very high levels of monocytic cytokines are produced, which spark a cascade of concomitant events such as tissue catabolism, vascular reactivity, and hypercoagulation with damaging effects on the host.[90,91] Anti-cytokine therapy (treatment to prevent cytokine production or activity) is currently being tested not only in major chronic inflammatory diseases including rheumatoid arthritis and ulcerative colitis, but also in critical infectious diseases such as septic shock and cerebral malaria.

IL-1β and TNF-α have similarities in function and may work synergistically. They both stimulate $PGE_2$ and collagenase production by fibroblasts. [92] They both stimulate bone and cartilage resorption.[93,94]

### *Prostaglandin $E_2$*

The cyclooxygenase is a major synthetic pathway relevant to human disease. The initial synthetic step involves the cleavage of arachidonic acid. Arachidonic acid is stored esterified in phospholipids of cell membranes. It is released from the cell membrane upon demand via phospholipase $A_2$.[95] Prostaglandins are one of the end products of the cyclooxygenase pathway. Prostaglandin $E_2$ is a prominent inflammatory mediator. It is formed in a variety of cells from prostaglandin $H_2$, which is synthesized from arachidonic acid by the enzymes cyclooxygenase or prostaglandin synthetase.[96,97] $PGE_2$ has been shown to have a number of biological actions, including vasodilation, [98] both anti- and proinflammatory activities,[99,100] modulation of sleep/wake cycles,[101] elevation of cAMP levels,[102] and thermoregulatory effects. It has also been shown that $PGE_2$ stimulates osteoclastic bone resorption[103-105] and collagenase production by activated macrophages.[106] $PGE_2$ can induce inflammatory changes in the periodontuim due to its ability to cause vasodilation and increased vascular permeability. It is hypothesized that $PGE_2$ is a likely contributing factor to the more advanced periodontitis seen in users of tobacco products. It has been implicated in progressive periodontitis. [105]

### *Transforming Growth Factor Beta (TGF-β)*

TGF-β is a stable, multifunctional polypeptide growth factor. It is a disulfide-linked, non-glycocylated dimer of two identical chains of 112 amino acids. It is stimulatory for cells of mesenchymal origin and inhibitory for cells of epithelial or neuroectodermal origin. TGF-β is an important modulator of the growth, differentiation, and activity of a number of different types of cells. It is produced by many cells including monocytes, but in higher concentrations in platelets and mammalian bone.

TGF-β is an important anti-inflammatory agent [107] It is produced locally at the site of resorption of bone and has been shown to initiate a new phase of bone formation. [108] In vitro, TGF-β has been shown to stimulate osteoblasts and to inhibit osteoclasts and thus may play a role in coordinating bone remodeling. [109,110] It has been shown that TGF-β can inhibit a range of IL-1 induced responses[111,112] and functions by reducing the constitutive or induced level of IL-1 receptors.[113]

**Lipopolysaccharide (LPS) Challenge**

A considerable array of bacterial components and products has been suggested, mainly on the basis of in vitro studies, as being responsible for the characteristic pathology of inflammatory conditions such as periodontal diseases.[114] These can be broadly divided into two groups, those that adversely affect host tissues directly (e.g., enzymes, end-products of metabolism) and those that stimulate the release of inflammatory mediators from host cells. Earlier studies appeared to be more concerned with bacterial products capable of directly inducing tissue destruction, [115-117] while more recent studies have tended to place greater emphasis on components able to alter the behavior of host cells.[118-120] For example, many early studies of the role of LPS and/or endotoxins in tissue destruction were primarily concerned with its cytotoxicity, [121-123] whereas it is now seen to exert its adverse effects as a consequence of its ability to stimulate cytokine release from macrophages and other host cells. [46,124,125]

Hahazawa et al. [45] first showed that *Porphyromonas gingivalis* LPS (PGLPS) induced IL-1 release from macrophages over the concentration range 1-50 μg/ml. Human peripheral blood mononuclear cells (HPBMC's) appeared to be more responsive to PGLPS with 0.1 μg/ml inducing IL-1 secretion, a finding confirmed by Yamazaki et al.[46] However, this may reflect the

response of murine and human LPS receptors to the bovine LPS binding protein/CD14-LPS complex, rather than to inherent differences in sensitivity to LPS itself. The ability of PGLPS to stimulate the release of IL-1 from murine macrophages has been confirmed in several subsequent studies.[126-128] McFarlane et al. (1990) found that HPBMCs from patients with periodontitis released significantly more IL-1β and TNF than those from periodontitis-free controls when stimulated with PGLPS (5 μ/ml).[129] In a study demonstrating the sensitivity of HPBMCs to PGLPS, Shapira et al. (1994) reported that TNF-α was released in a dose-related manner over the concentration range of 0.1 to 100 μg/ml with statistically significant release at 0.1 μg/ml.[43] The dose response was, in fact, very similar to that obtained using LPS from *Escherichia coli*. The reason for the increased potency of this LPS preparation compared with that found in other studies is not known.

McFarlane et al. (1990) showed that the LPS from *Actinobacillus actinomycetemcomitans* (*Aa*)stimulated peripheral blood monocytes from periodontitis patients to release significantly more IL-1β than control subjects.[129] Shapira et al. (1994) found that LPS stimulation resulted in the dose-dependent secretion of higher levels of $PGE_2$ and TNF-α by monocytes from localized aggressive periodontitis (LAP) patients compared to patients with severe chronic periodontitis.[43]

**Periodontal infection modulation of systemic health**

There is an association between periodontal disease and some systemic conditions such as cardiovascular disease,[51,130-134] and preterm delivery leading to low birth weight in pregnant women.[135] There might be a common underlying aspect of the host response that causes susceptibility of individuals to periodontal disease and these systemic complications. A macrophage/monocyte phenotype might be one such example. It is also possible that the presence

of periodontal infection serves as a reservoir of biological response modifiers such as LPS, $PGE_2$, TNF-α, and IL-1, which have systemic effects.

**Smokeless Tobacco (ST)**

Smokeless tobacco (ST) usage is associated with gingival recession and attachment loss.[136-138] The way by which ST exerts these effects is unknown; the chemical and/or mechanical irritating properties of ST are presumably responsible. ST contains numerous compounds such as nicotine and nitrosamines, which are local irritants.[139] In theory, irritation from ST components may stimulate the production of inflammatory mediators whereby leading to the observed tissue alterations in ST users. Arachidonic acid metabolites and various cytokines, such as IL-1β might be possible etiology of ST-induced lesions.

ST extract contains numerous chemicals. Although ST products vary considerably in their compositions, nicotine is the common element in all of these products. In a study by Bernzweig et al. (1998) on the effect of nicotine and ST on peripheral blood mononuclear cells (PBMC), it was shown that 1% ST alone induced a 3.5-fold increase in $PGE_2$ compared to control conditions.[140]

***ST and LPS***

Both ST and LPS are capable of stimulating monocyte secretion of $PGE_2$ and IL-1ß. In a study by Payne et al. (1994), it was shown that ST modulates the LPS-mediated monocyte response, potentiating $PGE_2$ release and resulting in either an increase or decrease in IL-1β release depending on the magnitude of the LPS response.[141] It was also shown that smokeless

tobacco was a more potent stimulator of $PGE_2$ than LPS from *P. gingivalis* and had an effect similar to LPS from *E. coli.*

Bernzweig et al. (1998) showed that the secretion of IL-1β by PBMC was not modulated by nicotine or ST alone relative to controls.[140] *P. gingivalis* LPS caused a 2.4-fold increase in IL-1β release compared to control. However, the addition of nicotine or ST to *P. gingivalis* LPS did not affect LPS-mediated IL-1β secretion.

**Nicotine and LPS**

Payne et al. (1996)[142] studied the effect of nicotine alone and in combination with LPS on monocyte secretion of IL-1β and $PGE_2$. The result showed that nicotine alone did not result in significant peripheral blood monocyte (PBM) secretion of $PGE_2$ and IL-1β above that of unstimulated cultures. However, $PGE_2$ release was potentiated 1.7-fold by the combination of *P. gingivalis* LPS and 10 μg/ml nicotine relative to *P. gingivalis* LPS alone. Prostaglandin $E_2$ release also was potentiated 3.5-fold by *P. gingivalis* LPS and 100 μg/ml nicotine relative to *P. gingivalis* LPS alone and 3.1-fold by *E-coli* LPS and 100 μg/ml nicotine relative to *E. coli* LPS alone. IL-1β secretion was lower for either LPS plus 100 μg/ml nicotine relative to LPS alone, although not significantly. Bernzweig et al. (1998) showed that 100 μg/ml nicotine resulted in approximately 7-fold increase in $PGE_2$ secretion.[140] In addition, 100 μg/ml nicotine and *P.gingivalis* LPS stimulated significantly more $PGE_2$ release than *P. gingivalis* LPS alone, but not more than nicotine alone. However, the addition of nicotine to *P. gingivalis* LPS did not affect LPS-mediated IL-1β secretion. Inhibition of osteogenesis in vitro by a cigarette smoke-associated hydrocarbon combined with *P. gingivalis* LPS was studied by Andreou et al.[143] It was

shown that smoke-derived aryl hydrocarbons and bacterial LPS might act additively to inhibit bone formation. The findings may explain, in part, why bone loss is greater and bone healing is less successful in smokers than nonsmokers with periodontal infection.

**Gingival Crevicular Fluid (GCF)**

It has been reported that IL-1β, [144,145] TNF- α, [146] and $PGE_2$ [147,148] are found in the gingival crevicular fluids of periodontitis patients and from clinically inflamed sites in human subjects. Of these, IL-1 has been studied in greatest detail. Due to its strong relationship with bone resorption, this cytokine has received considerable attention as a potential marker for active periodontal tissue destruction. IL-1β concentrations increase significantly during episodes of periodontal inflammation. [145,149] Cross-sectional studies have indicated that the levels of IL-1β are increased at periodontitis sites compared to gingivitis and healthy sites. [144,145] To date, there have not been enough longitudinal studies investigating the relationship between IL-1 levels in gingival crevicular fluid to make any conclusive statement regarding its usefulness as a diagnostic marker for periodontal disease activity.[151] Elevated $PGE_2$ levels have been noted in the gingival crevicular fluid from patints with localized aggressive periodontitis compared to patients with chronic periodontitis.[152]

It was shown by Boström et al. (1998) that TNF-α in GCF was significantly increased in both current and former smokers in treated and untreated patients with periodontal disease.[153] TNF-α was significantly increased in current smokers compared to nonsmokers. In contrast, the level of IL-6 was not influenced by smoking (Boström et al. 1999). [154]

A study was done to evaluate the levels of IL-1β, TNF-α, and neutrophil elastase activity in peri-implant crevicular fluid.[155] It was shown that the peri-implant crevicular fluid of implants

with inflamed gingiva had higher levels of lL-1β and neutrophil elastase activity than implants with non-inflamed or slightly inflamed gingiva. The peri-implant crevicular fluid of implants in smoker patients had significantly lower neutrophil activity and IL-1β levels, and significantly greater TNF-α levels than the peri-implant crevicular fluid of implants in nonsmokers.

**Nicotine Concentration in Plasma, GCF and Saliva**

According to Ryder et al. (1998), nicotine was not detected in the GCF of smokers; but immediately after smoking, the level of nicotine increased to 5961 ng/ml. [17]

Salivary concentrations of nicotine range from 73- 1560 μg/ml in ST users [156] and up to 1.3 μg/ml in smokers.[157] During smoking itself, nicotine concentrations in the oral cavity may reach even higher levels. Ryder et al. (1998) found a mean concentration of 109 ng/ml in the saliva of smokers; immediately after smoking marked elevation to 1821 μg/ml was detected. [17]

Plasma levels of nicotine in ST users and smokers are relatively low, with mean levels reported to be between 15 and 36 ng/ml. [158.159.160] Plasma levels in ST users and smokers range from 22 to 73 μg/ml and have been reported to be as high as 76 μg/ml, respectively.[159]

**Purpose**

The purpose of this study was to investigate the effect of chronic and acute exposure to whole smoke on production of IL-1β, TNF-α, $PGE_2$, and TGF-β released by adherent peripheral blood mononuclear cells (monocytes and lymphocytes). Monocytes and lymphocytes isolated from smokers' blood were chronically exposed to low levels of tobacco smoke in the bloodstream, which may affect cytokine release. This exposure has been commonly termed "chronic smoke exposure". However, during smoking, monocytes and lymphocytes in the mouth,

airways and lungs are exposed to short-term acute levels of smoke at much higher levels. [8,9,17,18] This type of exposure is called "acute smoke exposure". It has been shown that levels of smoke exposure in an in vitro smoke box system are comparable to levels seen in the oral cavity during acute smoke exposure. [17,18] In this study, this smoke box system was used to determine the effects of acute smoke on cytokine release from adherent peripheral blood monocytes and lymphocytes. The significance of this study is that it is the first investigation of the effect of chronic and acute whole smoke on mononuclear cells and their secretion of inflammatory mediators.

## Null Hypothesis

There is no difference between the amount of interleukin-1β, prostaglandin $E_2$, tumor necrosis factor-α, and transforming growth factor-β released by peripheral blood mononuclear cells (monocytes and lymphocytes) obtained from smokers versus nonsmokers when the cells are subjected to acute smoke exposure in vitro.

## Material and Methods

This protocol was approved by the UCSF Committee on Human Research.

### Cell separation and Culture

Peripheral blood was isolated from 8 medically healthy current smokers (more than one pack per day) with no reported serious medical conditions such as pulmonary or cardiovascular diseases (4 males, 4 females, mean (-/+ SD) age=46.00-+ 12.60 years) and 8 medically healthy nonsmokers (3 males, 5 females, mean (-/+ SD) age 34.00+-13.24 age). Subjects were included in the study if they did not take long-term anti-inflammatory drugs, which could alter neutrophil function, and did not have a systemic condition such as diabetes or immunosuppression, which

could markedly alter mononuclear blood cell function. After obtaining informed consent, approximately 60 ml of peripheral blood was collected from each subject by venipuncture into heparinized tubes and diluted 1:1 with phosphate-buffered saline (PBS). The mononuclear cells were separated by Ficoll-Hypaque sedimentation[161] layered over 10 cc of Histopaque 1077 (Sigma Diagnostics, St. Louis, MO), and centrifuged for 10 minutes at 700g. Histopaque-1077 is a solution of polysucrose and sodium diatrizoate adjusted to a density of 1.077 +/- 0.001 g/ml. This medium facilitates rapid recovery of viable mononuclear cells from granulocytes and erythrocytes. The mononuclear leukocyte fraction, which formed a cloudy section in the middle of the tube, was drawn off into PBS; this fraction then was diluted with 50 cc PBS. 4 cc-sterilized water was added to the pellet of mononuclear cells to lyse any residual erythrocytes followed by immediate addition of 1cc 5xPBS. After dilution with PBS, the suspension was centrifuged for 10 min (700g). The pellets of mononuclear cells were removed and resuspended in RPMI 1640 media with 25 mM HEPES. An aliquot of the cells was counted in a hemocytometer, and the remaining cells were plated in 24-well culture dishes at a concentration of $4 \times 10^6$ cells per well. Each well contained 1 cc of culture media with suspended mononuclear cells. The mononeuclear blood cells then were incubated for 90 minutes at 37°C in a humidified atmosphere containing 5% $CO_2$ to allow adherence of mononuclear blood cells to the bottom of each well. Non-adherent cells were removed by aspiration and the wells washed three times with PBS. A volume of 1 mL of RPMI 1640 medium with 25 mM HEPES was then added to each well.

**Smoke exposure and incubation**

The adherent mononuclear blood cells in culture wells were exposed to in vitro smoke for 1, 2, and 5 minutes using an enclosed smoke box or not exposed to further in vitro smoke (baseline controls). In this smoke box system as previously described[17,18] , cigarette smoke was generated from the unfiltered lit end of class A filtered cigarettes. The source of cigarette smoke was separated from the plates containing mononuclear cells by 3 layers of a 0.5 mm plastic mesh screen to prevent large particles of matter from entering the mononuclear cells suspension. In addition, 2 layers of the same screen were placed over the plate for the same purpose. Smoke was introduced into the chamber through intermittent injection of air through the cigarette (puffs) at the rate of 6 puffs per minute. The smoke was allowed to circulate over the mononuclear blood cells by introducing a gentle system of air at one end of the chamber and pumping the air out by vacuum through the other end of the chamber at a pressure of ± 5 psi. During the time of smoke exposure, the chamber was placed on a rotary shaker platform operating at approximately 30 rotations per minute. After exposure, the culture dishes were then incubated for 24 hours without further smoke exposure at 37°C in the humidified atmosphere containing 5% $CO_2$.

**Immunoassays**

Following incubation, cell culture supernatants were drawn from each well and analyzed by enzyme-linked immunoassay (ELISA) for measuring the levels of IL-1β, $PGE_2$, TNF-α, and TGF-β. The remaining adherent cells were stored at 4°C for DNA quantification to determine the relative cell numbers per well.

***Immunoassay for IL-1β, TNF-α, and TGF-β***

Levels of IL-1β, TNF-α, and TGF-β in culture supernatants were determined by commercially available enzyme immunoassay kits (Quantikine, R&D Systems Inc., Minneapolis, MN, USA). These assays employ the quantitative sandwich enzyme immunoassay technique (ELISA). Briefly, plates were precoated with a specific monoclonal antibody to each of these cytokines. Subsequent stages included incubation with standard or sample, a wash to remove any unbound antibody-enzyme reagent, addition of a substance to the wells and color development in proportion to the amount of IL-1β, TNF-α, or TGF-β bound in the initial step. The color development is stopped and the intensity of the color is measured.

Plates were read at 450 nm and 540 nm. The readings at 540 nm were subtracted from the readings at 450 to correct for optical imperfections in the plate. The sensitivities for these assays were IL-1β: 1 pg/ml; TNF-α: 4.4 pg/ml; and TGF-β: 7 pg/ml. Calibration curves with known quantities of each of these three cytokines were conducted in duplicate. Quantifications of the three cytokines for each subject at each experimental time point were conducted in triplicate.

***Immunoassay for IL-1β***

200 µl of standard (recombinant human IL-1β, with six different concentrations range: 125-3.9 pg/ml) or sample were added to each well followed by incubation for 2 hours at room temperature. Aspiration and wash using wash buffer (buffer surfactant) was performed three times. 200 µl of IL-1β conjugate (polyclonal antibody against IL-1β conjugated to horseradish peroxidase) was added to each well followed by incubation for 1 hour at room temperature. Aspiration and wash were repeated. 200 µl of substrate solution (mixture of stabilized hydrogen peroxide and chromogen) was added to each well followed by incubation for 20 minutes at room

temperature. Then, 50 µl of stop solution (2 N sufuric acid) was added to each well. The optical density of each well was determined within 30 minutes.

***Immunoassay for TNF-α***

50 µl of assay dilutent (buffered protein base) was added to each well. 200 µl of standard or sample was added to each well followed by incubation for 2 hours. Aspiration and wash with wash buffer (buffered surfactant) was performed twice. 200 µl conjugate (polyclonal antibody against TNF-α conjugated to horseradish peroxidase) was added to each well followed by one-hour incubation. Aspiration and wash were repeated. 200 µ of substrate solution (mixture of stabilized hydrogen peroxide and chromogen) were added to each well. 50 µl of stop solution (2 N sulfuric acid) to each well. The optical density of each well was determined within 30 minutes with wavelength of 450 nm and 540 nm. Readings from 540 nm were subtracted from 450 nm.

***Immunoassay for TGF-β***

To activate latent TGF-β to the immunoactive form, HCl was added to samples followed by incubation for 10 minutes at room temperature. Neutralization of the acidified sample was performed by adding NaOH/HEPES. 200 µl of standard (recombinant human TGF-$\beta_1$ in different concentration range 1000-31.2 pg/ml) or activated samples were added to each well. Incubation was provided for three hours at room temperature. Wells were aspirated and [1] washed twice with wash buffer. 200 µl of TGF-$\beta_1$ conjugate (polyclonal antibody against TGF-$\beta_1$ to horseradish peroxidase) was added to each well followed by incubation for 1.5 hours at

stabilized hydrogen peroxide and chromogen) was added to each well followed by incubation for 20 minutes at room temperature. 50 µl of stop solution (2 N sulfuric acid) was added to each well and the optical density of each well was determined within 30 minutes at 450 nm and 540 nm. Substraction at 540 nm from 450 nm was done to correct for optical imperfections in the plate.

***Immunoassay for $PGE_2$***

Levels of $PGE_2$ in culture supernatants were determined by a commercially available enzyme immunoassay kit (Quantikine, R&D Systems Inc. Minneapolis, MN, USA). This assay is based upon competition between the unknown concentrations of free $PGE_2$ in the samples with a known concentration of alkaline phosphatase-labelled $PGE_2$ for the limited number of $PGE_2$ specific mouse antiserum binding sites in each of the assay wells. Briefly, the technique entailed adding sample or standard with mouse monoclonal antibody to human $PGE_2$ to ELISA plates precoated with goat anti-mouse antibody. A subsequent step was the addition of alkaline phosphatase-labelled $PGE_2$ ($PGE_2$ conjugate). During the incubation, the mouse monoclonal becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution (para-nitrophenyl phosphate) was added to the wells to determine the bound enzyme activity. A stop solution (trisodium phosphate) was added followed by immediate reading of absorbance at 405 nm and 54 nm. The intensity of the color is inversely proportional to the concentration of $PGE_2$ in the sample. Calibration curves with known quantities of $PGE_2$ were conducted in duplicate. Quantitations of $PGE_2$ for each subject at each experimental time point were conducted in triplicate.

**DNA Assay**

The DNA content of the adherent cells in culture wells was assayed as described by Shapira et al. (1992).[162] Each well was sonicated for 20 seconds. Bisbezimidazole or 4,6-diamidino-2-phenylindole (DAPI) was added to a final concentration of 1μg/ml and the DNA content was measured using luminescence spectrophotometer at an excitation wavelength of 365 nm and an emission wavelength of 450nm. Following addition of DAPI, the fluorescence reaction was recorded immediately. The DAPI-DNA complex is stable at room temperature for several hours, and it is not readily photodissociated by the spectrofluorometer[163] DAPI interacts with high-molecular weight DNA by specific binding to adenine-thymidine base pairs (A-T), thereby making the reaction sensitive to the A-T content of the DNA. Since A-T content of DNA is species-specific, calibration of the system with appropriate DNA preparation is an important consideration. Human placental DNA served as the standard. The purpose of this DNA assay was to assess indirectly whether there were marked differences in adherent mononuclear blood cell numbers between different time-points, and to adjust the concentration values where there were significant differences in DNA content. Emission intensities of known quantities of human placental DNA were used for calibration of values of the experimental samples between baseline and 1, 2, or 5 minutes of smoke exposure. As there were no differences in DNA content between baseline and 1,2,or 5 minutes of smoke exposure, no adjustments to cytokine concentrations relative to cell numbers were performed.

## Statistics

For statistical analysis, each cytokine concentration for each subject at each time point was derived from the mean of the triplicate samples from each time point. These resulting means

from each subject at each time point were considered the individual "n" values. For differences in cytokine concentrations between smokers and nonsmokers at each time point, a repeated measures analysis of variance (ANOVA) was used. For differences of cytokine concentrations at different time points within the smoking and nonsmoking groups, the paired Student's t-test was used within each subject. Results were considered statistically significant at $p< 0.05$.

## Results

### IL-1β

The mean secreted levels of IL-1β from mononuclear blood cells at baseline (time zero) and after exposure to 1, 2, and 5 minutes of in vitro smoke in the smoke box system (acute smoke exposure) were consistently higher among the 8 smokers when compared to the 8 nonsmokers (figure 1). The mean values of secreted IL-1β in smokers compared to nonsmokers were 90% higher at baseline ($p<0.05$, repeated measures ANOVA), 88% higher at 1 minute (p=. 0.052), 44% higher at 2 minutes (p=0. 073), and 32% higher at 5 minutes (p=0.36). In the nonsmoking group, there was a mean increase in IL-1β secretion of 76% from baseline to 5 minutes ($p< 05$, paired t-test). In the smoking group, there was a mean increase in IL-1β secretion of 32% from baseline to 5 minutes ($p>0.05$). When comparing the distribution of secreted IL-1β values for individual subjects within the smokers and nonsmokers group, there was a considerable range of values for each time point in each group as shown in the box plots of the distribution of values in Figure 2. For example, at baseline, the secreted IL-1β values for individual subjects ranged from 3.39-12.89 pg/mL of supernatant for nonsmokers and 3.53-20.35 pg/mL for smokers. While the extreme range of values of both smokers and nonsmokers were

comparably broad, the range of values within the 50$^{th}$ percentile around the median value was considerably greater in the smoker group.

**TNF-α**

The mean secreted levels of TNF-α from mononuclear blood cells at baseline (not exposed to further in vitro smoke) and at all times (exposed to 1, 2, and 5 minutes of in vitro smoke, acute smoke exposure) were consistently higher in nonsmokers compared to smokers (figure 3). Specifically the mean values of secreted TNF-α in nonsmokers compared to smokers were 43% higher at baseline, 75% higher at 1 minute, 65% higher at 2 minutes, and 53% higher at 5 minutes. None of these differences were statistically significant. In the nonsmoker group, there was a 29% higher mean secretion of TNF-α at 2 minutes when compared to baseline which was not significant. This rise was due in part to a greater range of values at this time point (figure 4 box plot). The mean secreted TNF-α values from smokers were essentially similar at baseline when compared to 1, 2, and 5 minutes of smoke exposure. While the extreme range of values of both smokers and non-smokers were comparably broad, the range of values within the 50$^{th}$ percentile around the median was considerably greater in the group of nonsmokers (figure 4).

**$PGE_2$**

The mean values between smokers and nonsmokers were similar at baseline and at 1, 2, and 5 minutes of smoke exposure (figure 5). At 5 minutes of smoke exposure, the mean $PGE_2$ values were 141% greater in nonsmokers and 94% greater in smokers when compared to baseline values. These differences were not significant in part due to the much wider spread of upper and lower values among the individual subjects at 5 minutes when compared to baseline (figure 6).

For example, although the range of values within the 50th percentile around the median value were similar in nonsmokers at baseline and at 5 minutes, the extreme range of values for nonsmokers was 93.5-665.0 PG/Ml of supernatants at baseline vs. 175.3-3396.0 pg/mL at 5 minutes (figure 6). A similar distribution of $PGE_2$ values was observed in smokers at baseline as compared to 5 minutes of smoke exposure.

### TGF-β

The mean level of TGF-β secreted from mononuclear blood cells was higher in smokers at baseline (not exposed to further in vitro smoke) and at all times (exposed to 1, 2, and 5 minutes of in vitro smoke) compared to nonsmokers (figure 7). The mean values of secreted TGF-β, in smokers compared to nonsmokers were 44% higher at baseline (NS), 32% higher at 1 minute (NS), 32% higher at 2 minutes (NS), and 36% higher at 5 minutes ($p<.05$). For both smokers and nonsmokers, there were no significant differences in mean secreted TGF-β values between baseline and at 1, 2, or 5 minutes of smoke exposure. The range of TGF-β values for individual subjects was generally greater in the smoking group when compared to the nonsmoking group (figure 8). This greater range of values in the smoking group included both a greater range in extreme values and a greater range of values in the 50th percentile around the median value.

## Discussion

In this study, the effects of in vitro smoke on cytokine secretion from smokers and nonsmokers were assessed. Other factors such as age and periodontal disease were not equalized between groups due to the small size of the study population. The conditions of in vitro smoke

exposure used in this study and in previous studies may be comparable to levels of smoke in the oral cavity, airways, and periodontal tissues during acute smoke exposure [8,7,16,17,32]

The level of IL-1β increases from baseline to 5-minute exposure in both smokers and nonsmokers. It shows that acute exposure to smoke increases secretion of IL-1β. The level of IL-1β was higher in smokers versus nonsmokers at baseline and at all times, with statistically significant differences at baseline. This indicates that chronic exposure to smoke causes elevation of IL-1β secretion. Elevation in the level of IL-1β, which also has been shown in previous studies, may be one of the factors for the increased severity and prevalence of periodontal disease in smokers (Offenbacher 1996; Schenkein 1999). [13,12]

The mean secreted levels of TNF-α from mononuclear blood cells at baseline and at all times were consistently higher in nonsmokers compared to smokers. The level of TNFα in both groups increased from baseline to 2 minutes followed by a fall from 2 minutes to 5 minutes. The fall could be explained by possible sublethal effect at 5 minutes or perhaps the secretion of TNF-α was exhausted with smoke exposure.

There was no significant difference in $PGE_2$ between smokers and nonsmokers at baseline. There was an increase from baseline to 5 minutes exposure. This shows that acute smoke causes elevation in $PGE_2$ secretion. In this study, the elevation of $PGE_2$ was primarily due to marked elevations after 5 minutes of smoke exposure in one smoking and one nonsmoking subject. Further studies on a larger group of subjects may yield a clearer statistical pattern.

The results show that in smokers mononuclear cells chronically produce higher levels of the potentially destructive inflammatory cytokine, IL-1β. Furthermore, levels of acute smoke exposure as seen in the mouth during smoking appear to enhance release of more IL-1β as well

as $PGE_2$ and TNFα in a time-related manner. These elevations were significant for IL-1β secretion in nonsmokers. It is possible that these elevations may contribute to the progression and severity of periodontal disease in smokers (Offenbacher 1996, Shenkein 1999).[13,12] But, a direct connection between smoking and elevated cytokines has not been definitely determined in periodontal diseases or in other systemic conditions such as cardiovascular disease and chronic obstructive pulmonary diseases. There are discrepancies in the literature with regard to the effect of smoke on the release of inflammatory cytokines.[140-142, 164-167] These studies have shown positive or negative or no effect on release of these cytokines by acute or chronic smoke exposure. This could be due to the differences in type of cells, groups of cells, or organ system used in these studies, or to the type of exposure to the tobacco products. For example, Wesselius et al.[164] and Wewers et al.[165] have reported decreased levels of cytokines from macrophages cultured from smoker's lungs vs. nonsmoker's lungs. However, such in vitro studies do not take into account the increased numbers of inflammatory cells in the lung, and the effect of other local tissue cells to the overall levels of these inflammatory mediators.[168] According to Carty et al.,[169] in the case of atherosclerosis, the smooth muscle cells of the vascular intimae may release cytokines when exposed to tobacco smoke. It is also shown that epithelial cells in the oral cavity when exposed to smokeless tobacco extracts may contribute to the secreted pool of inflammatory mediators.[170] In this study a mixed culture of monocytes and lymphocytes was used which may give a more complete picture of cytokine secretion than monocytes alone,[140] but does not include the contribution from other host response cells such as neutrophils, macrophages and resident cells of periodontium such as epithelial cells, fibroblasts.

It has been shown in the literature that smoke products can affect the secretion of cytokines when stimulated by lipopolysaccharides from selected bacteria.[164,140] The results are

contradictory depending on the time of smoke and LPS exposure, and type of cell culture studied. Furthermore, Hasday et al.[171] has shown that cigarette smoke itself contains significant amounts of LPS. This finding makes it difficult to study the separate effects of tobacco products and LPS in cytokine secretion. Furthermore, studies that have been done on the effect of nicotine alone do not take into account the effect of about 2000 other substances in tobacco that may contribute to the altered release of cytokines. For example, it has been shown that while nicotine did not have an effect on cytokine secretion by macrophages, hydroquinones found in smoke could suppress the release of IL-1β and TNF-α.[172] It should be mentioned that in that particular study, monocytes were exposed to these substances for 3 hours vs. the maximum 5-minute exposure of mononuclear cells in the present study.

From this study and others, it is evident that chronic and acute levels of smoke can potentially elevate cytokines such as IL-1β, TNF-α, and $PGE_2$ in periodontal tissues and in other tissues and organs of the body exposed to smoke such as lung. Cytokines secreted in the lung and oral cavity can enter the blood stream and cause an increase in incidence and severity of the systemic diseases. For example, increased levels of TNF-α and/or IL-1β from smoke-exposed periodontal tissue and airways could lead to increased insulin resistance with exacerbation of diabetic mellitus, decreased levels of high density lipoproteins and elevation of low density lipoproteins.[13,134,173] These could lead to concomitant development of atherosclerosis, and elevation of C-reactive proteins which are risk indicators for a variety of inflammation related systemic diseases. Therefore, acute smoke not only can play a role in the progression of periodontal disease by elevating the cytokine production but may also play a role in possible relationship between periodontal diseases and systemic diseases. Many studies have shown that in patients with periodontal disease there is an increase in the local levels of IL-1β and $PGE_2$.

These inflammatory mediators could enter the blood stream and affect systemic conditions such as cardiovascular problems and low birth weight.

The factors that can affect the amount of secretion of cytokines are the age of individuals and the feedback pathways among cytokines. In the present study, the mean age of the smoking population was 12 years older than nonsmokers. This age discrepancy may be an important variable in the reported differences between cytokine secretions in smokers vs. nonsmokers as shown by some studies.[74,164,168] There is positive and negative feedback among the cytokines. For example, elevated prostaglandin levels may suppress the secretion of IL-1β, while IL-1β and TNF-α may reciprocally induce each other's secretion.[13] Furthermore, smoke products may enhance the destructive effects of inflammatory cytokines by suppressing the expression of IL-1β receptor antagonists in periodontal tissues.[174,175] Kunkel et al.[176] have shown a regulatory interplay between IL-1β and $PGE_2$. IL-1 upregulates $PGE_2$ synthesis by macrophages. Conversely, increased $PGE_2$ concentrations inhibit IL-1 production. Furthermore, the addition of cyclooxygenase-inhibiting drugs can increase IL-1 synthesis by decreasing arachidonic acid metabolites such as $PGE_2$. Therefore, IL-1 can regulate its own production through a self-induced inhibitor, $PGE_2$.[176] $PGE_2$ modulates IL-1 levels by a negative feedback mechanism, it is conceivable that, in the subjects in whom depressed IL-1β release was observed, this phenomenon might have been due to inhibition of IL-1β synthesis by $PGE_2$.

In this study, the level of TGF-β did not seem to be associated with acute exposure. But, the level of TGF-β was higher in smokers compared to nonsmokers at all time points. This shows that chronic exposure could cause an elevation of TGF-β. This could be interpreted as a compensatory mechanism for the destruction caused by the elevated inflammatory cytokines. TGF-β has a reparative function that is increased in tissue in response to smoking to increase turn

over and repair mechanisms against damage from smoke [171] Whether these elevations of TGF-β from peripheral blood mononuclear cells have a beneficial or detrimental effect on the periodontal tissues has yet to be determined.

Future studies are necessary to further investigate the effect of nicotine and whole smoke on other cell types and on studying the mechanism(s) by which nicotine and whole smoke modulates mononuclear secretory response.

## Conclusion

1. Chronic and acute levels of smoke can potentially elevate inflammatory cytokines such as IL-1β, TNF-α, and $PGE_2$ in periodontal tissues and in other tissues and organs of the body exposed to smoke.
2. Chronic exposure to smoke could cause an elevation of TGF-β. This could be a compensatory mechanism for the destruction caused by the elevated inflammatory cytokines.
3. Future studies are necessary to investigate the effect of nicotine and whole smoke on all cell types and the mechanisms of their action.

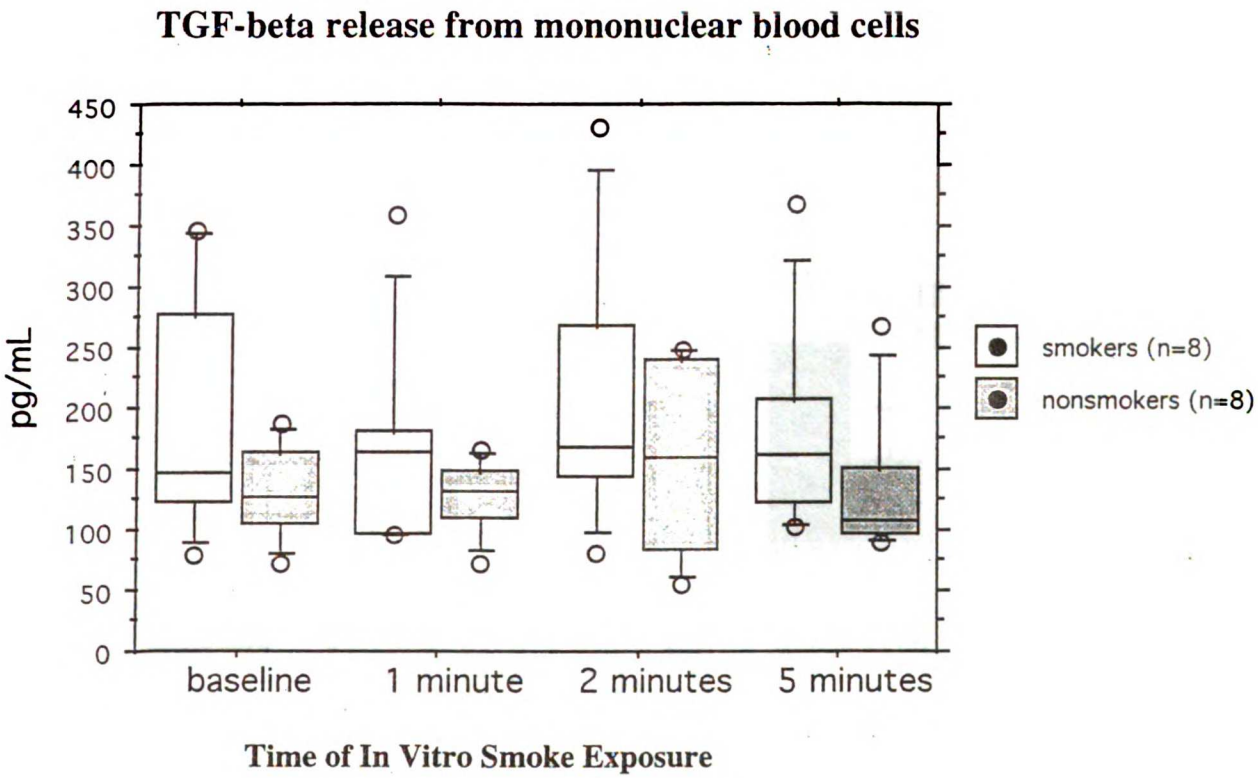

TGF-beta release from mononuclear blood cells
pg/mL
450
400
350
300
250
200
150
100
50
0
baseline
1 minute
2 minutes
5 minutes
Time of In Vitro Smoke Exposure
smokers (n=8)
nonsmokers (n=8)

Figure 8

## TGF-beta release from mononuclear blood cells

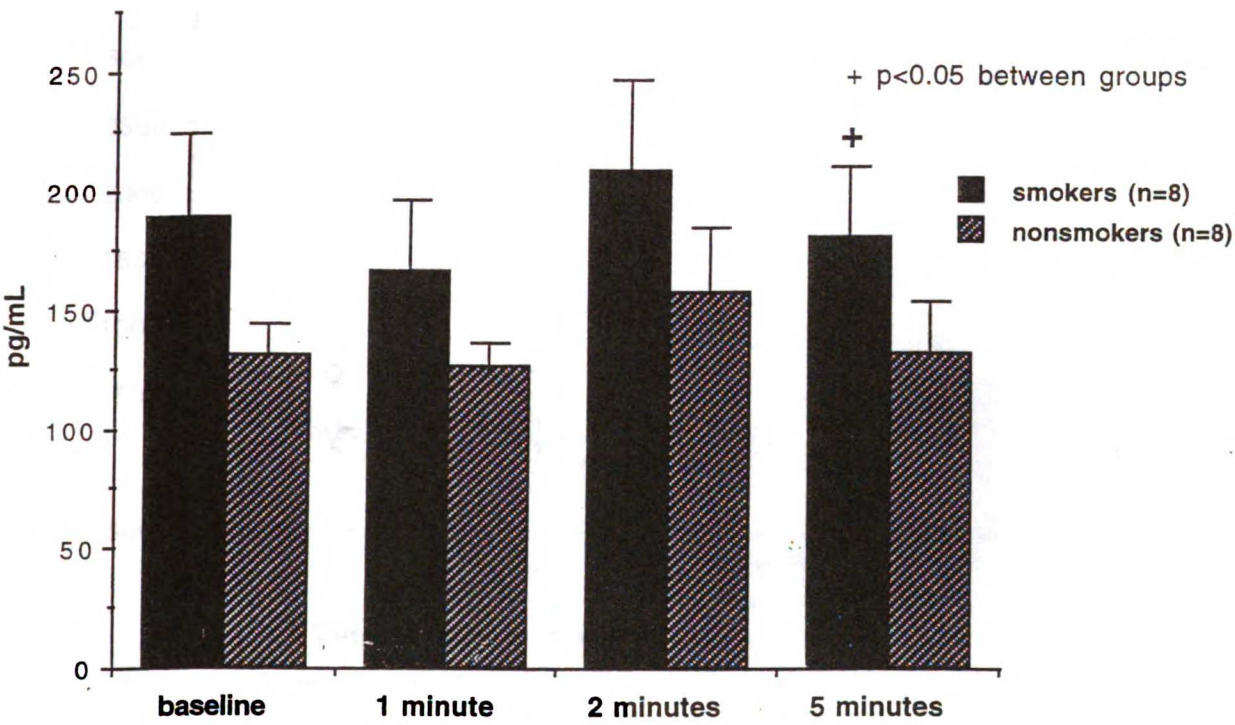
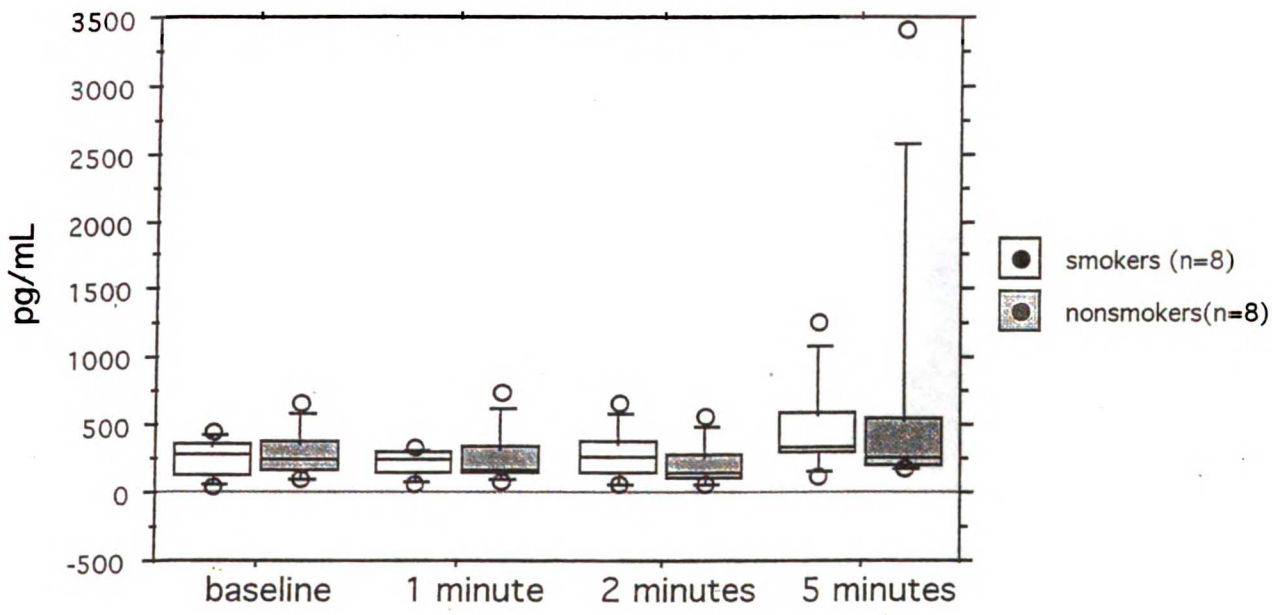



Figure 7

**PGE-2 release from mononuclear blood cells**




**Time of In Vitro Smoke Exposure**

Figure 6

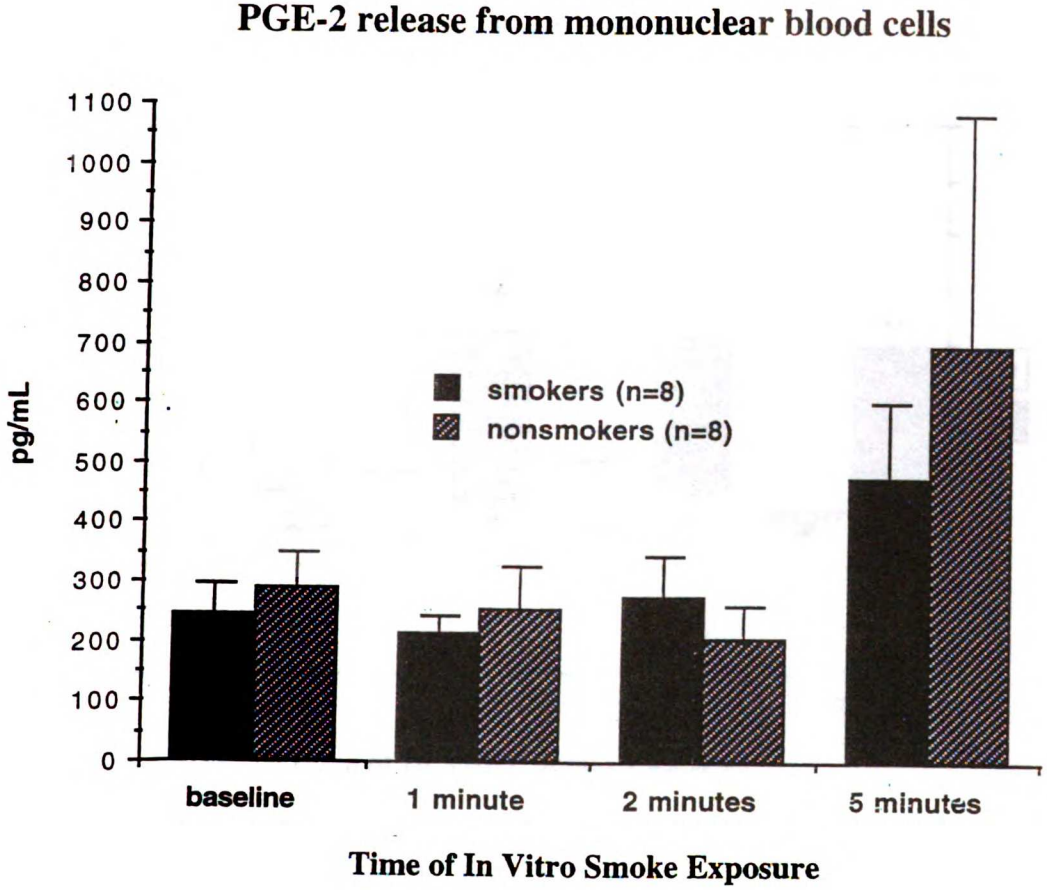

PGE-2 release from mononuclear blood cells
1100
1000
900
800
700
600
500
400
300
200
100
0
pg/mL
smokers (n=8)
nonsmokers (n=8)
baseline
1 minute
2 minutes
5 minutes
Time of In Vitro Smoke Exposure



Figure 5

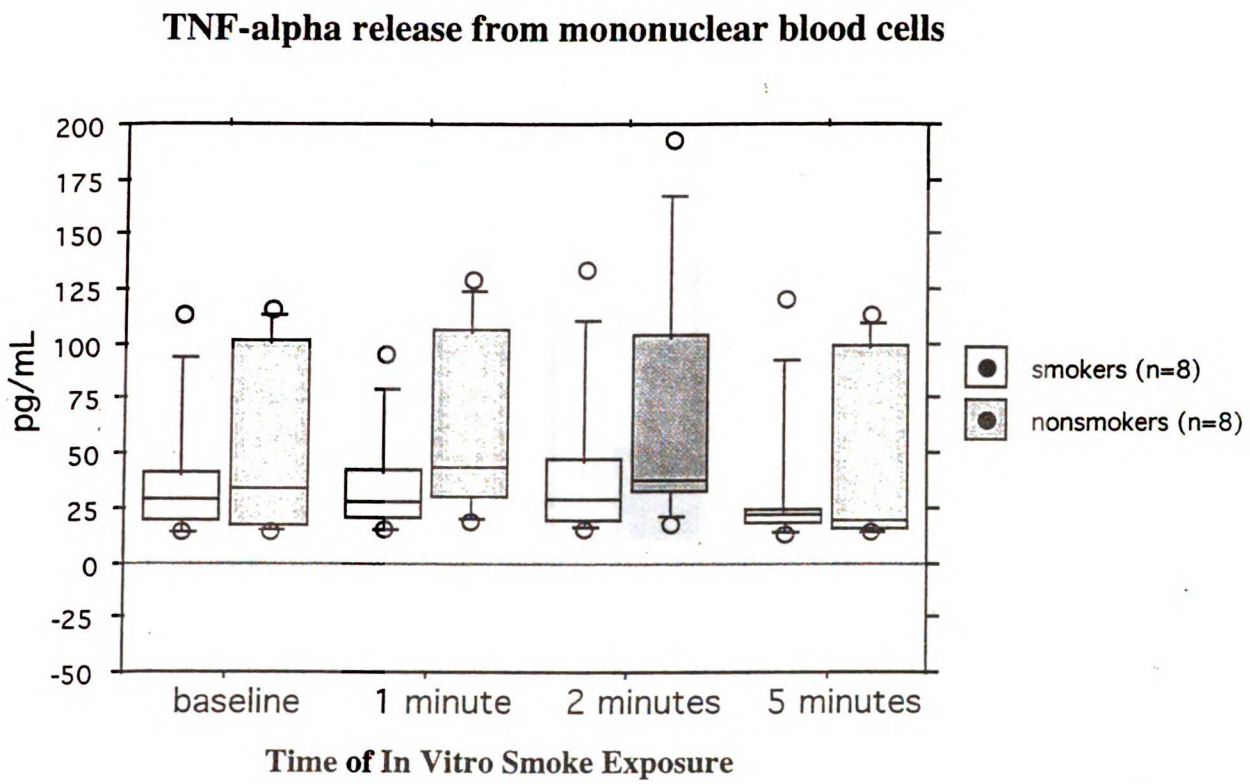

TNF-alpha release from mononuclear blood cells
200
175
150
125
100
75
50
25
0
-25
-50
pg/mL
baseline
1 minute
2 minutes
5 minutes
Time of In Vitro Smoke Exposure
smokers (n=8)
nonsmokers (n=8)

Figure 4

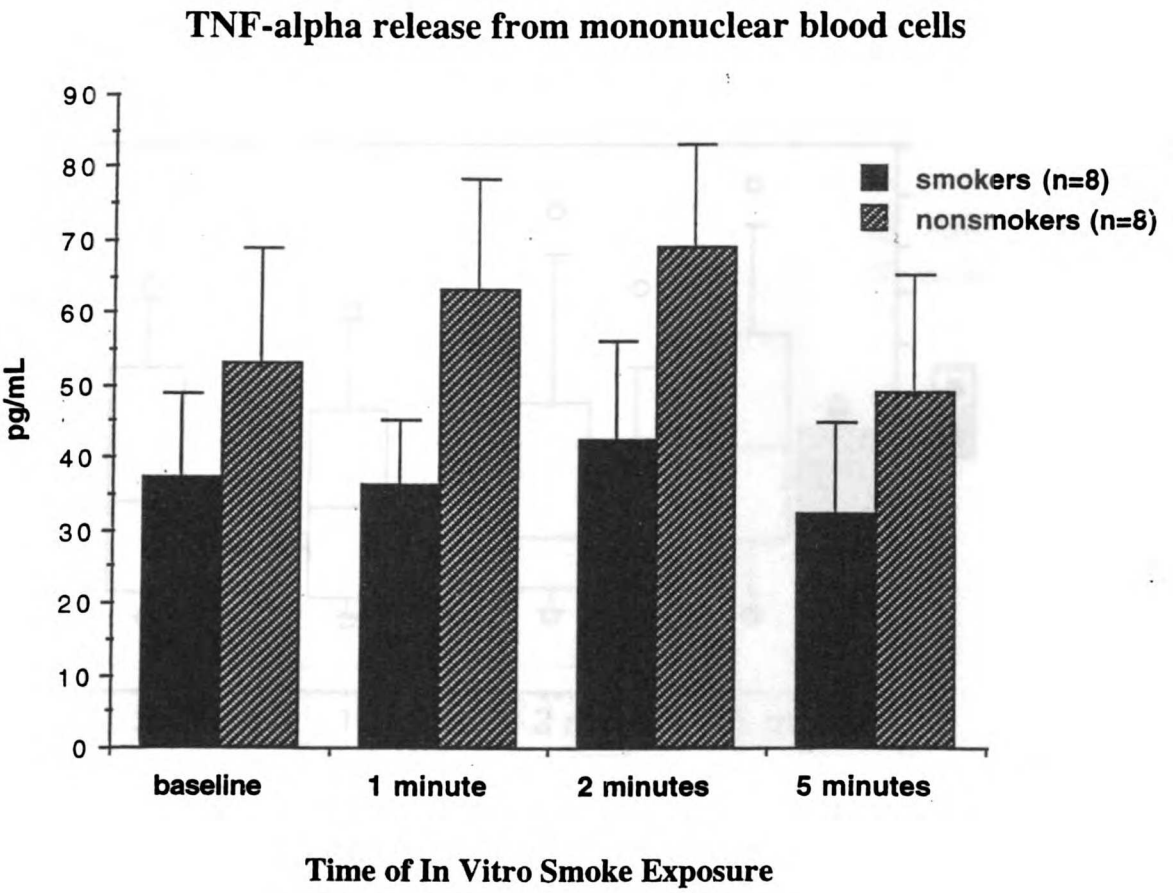

TNF-alpha release from mononuclear blood cells
90
80
70
60
50
40
30
20
10
0
pg/mL
smokers (n=8)
nonsmokers (n=8)
baseline
1 minute
2 minutes
5 minutes
Time of In Vitro Smoke Exposure



Figure 3

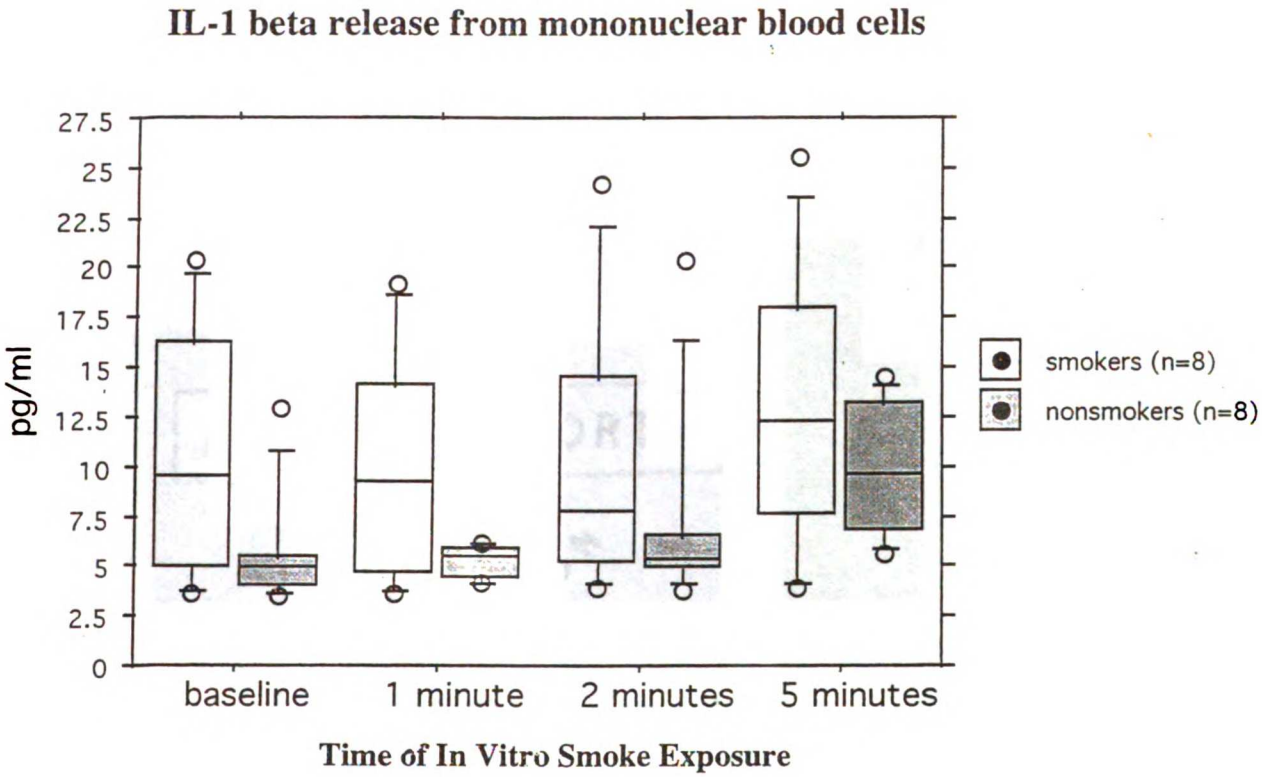

IL-1 beta release from mononuclear blood cells
pg/ml
27.5
25
22.5
20
17.5
15
12.5
10
7.5
5
2.5
0
baseline
1 minute
2 minutes
5 minutes
Time of In Vitro Smoke Exposure
smokers (n=8)
nonsmokers (n=8)

Figure 2

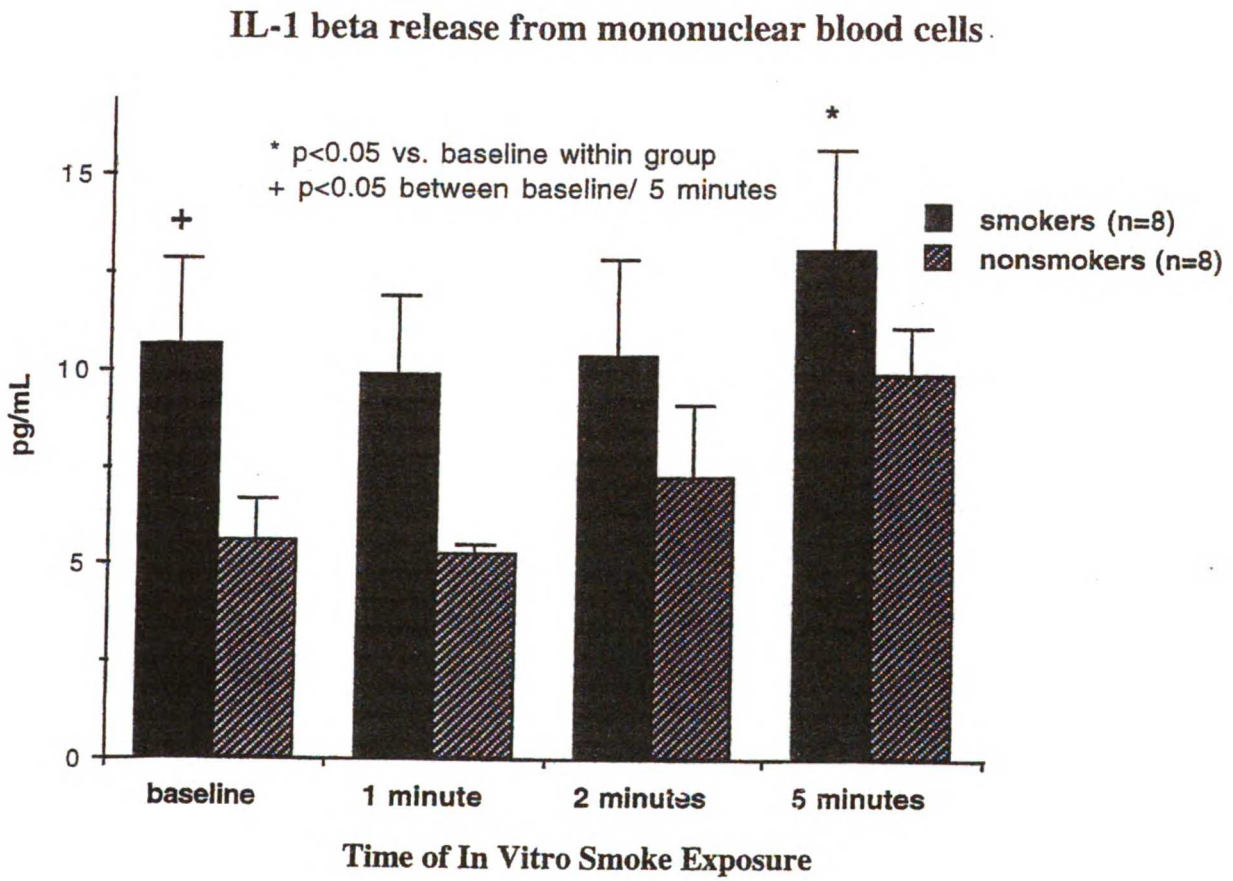

IL-1 beta release from mononuclear blood cells
* p<0.05 vs. baseline within group
+ p<0.05 between baseline/ 5 minutes
smokers (n=8)
nonsmokers (n=8)
pg/mL
15
10
5
0
+
*
baseline
1 minute
2 minutes
5 minutes
Time of In Vitro Smoke Exposure

Figure 1

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