Barts and The London
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Mechanism of Ciclosporin-induced gingival hyperplasia

An *in vitro* study of the effect of ciclosporin on human gingival fibroblasts and oral keratinocytes

by

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Abstract

Objectives:

Ciclosporin (CsA) is a potent and effective immuno-suppressive drug, but its use is associated with the development of gingival hyperplasia in up to 70% of patients.

The mechanism underlying this side effect is unknown and the aim of this *in vitro* study was to investigate the direct effect of CsA on oral epithelium and connective tissue and to explore whether oral bacterial products modified the response.

Methods:

3 human oral and gingival keratinocyte cell lines (OKF6, TR146 and FIBS) and a human gingival fibroblast cell line (HGF) along with reconstructed human gingival and oral epithelial models (RHGE and RHOE respectively, SkinEthic) were used in the study.

Cells/tissues were pre-stimulated for 24h with bacterial supernatants (*P. gingivalis* (Pg), *A. actinomycetemcomitans* (Aa), *F. nucleatum* (Fn), and *P. intermedia* (Pi) at 1/100 or 1/10 respectively and then exposed to a combination of CsA (2000 or 250 ng/ml) and bacterial supernatant for further 24h or 48h.

Cell viability, cytokine/ chemokine (IL-1α; IL-6; IL-8) release; cell cycle and proliferation markers (DNA synthesis, cyclin B1, cyclin D1, CCNB1, and CCND1) and apoptosis (Bax, Bcl-2 protein and gene, and Fas-L gene) were assessed using MTT assay, ELISA, FACs analysis, quantitative PCR. Cell cycle analysis, DNA synthesis and expression of cyclins D1 and B1 in cell lines were evaluated simultaneously.

Results:

CCNB1 and CCND1 were significantly up-regulated in all RHGE cultures exposed to any of the combinations applied compared to controls (CCNB1: CsA+A.a; 1.543± 0.039; CsA; 0.703± 0.032; A.a; 0.669± 0.002; untreated control; 1± 0.047)(CCND1; CsA+A.a; 3.41± 22 versus CsA; 1.046± 0.079; A.a; 2.127± 0.22; and untreated

control; 1± 0.106). FACS analysis of monolayer cultures showed an apparent increase in proliferation rate, illustrated by DNA synthesis, of FIBS, OKF6, and HGF consistently with the combination (P.i plus 2000ng/ml CsA) compared to controls. CsA at 2000ng/ml caused a significant increase in cytokine release from both keratinocyte and fibroblast cell lines. Furthermore, bacterial products in combination with CsA had a synergistic effect on IL-6 and IL-8 release from HGF but had a little effect on cytokine release from keratinocytes. Conversely, the combined treatments significantly decreased IL-8 in RHOE.

Conclusion:

The results suggest that a clinically relevant dose of CsA (2000ng/ml) along with bacterial products stimulate the proliferation of gingival keratinocyte and HGF by activating the cell cycle and DNA replication. Thus, the presence of specific periodontal microorganisms may be important in the development of the condition.

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List of Abbreviations

A.a	*Actinobacillus actinomycetemcomitan*s
7-AAD	7-aminoactinomycin D
ANOVA	analysis of variance
Bax	Bcl2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
BSA	Bovine serum albumin
BrdU	5-Bromo-2'-deoxy-uridine
CCNB1	Cyclin B1 gene
CCND1	Cyclin D1 gene
CsA	Ciclosporin
CYP3 A4	Cytochrome P450 3A4
C4S	Chondroitin-4- sulphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Edu	5-ethynyl-2´-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
Fas-L	Fas ligand
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FIBS	Human gingival keratinocytes
F.n	*Fusobacterium nucleatum*
FSC	Forward scatter
G0	Quiescence phase
G1	Gap/growth phase 1
G2	Gap/growth phase 2
GAG	Glycosaminoglycan
GCF	Gingival Crevicular Fluid
HCl	Hydrochloric acid
HGF	Human gingival fibroblasts
HLA	Horizontal Long Axis
IL-	Interleukin
KGFR	Keratinocyte growth factor receptor
LDH	Lactate Dehydrogenase
mRNA	Messenger ribonucleic acid
mg	milligram
ml	millilitre
MMP	Matrix metalloproteinase
MTT	(tetrazolium salt 3[4,5-3 dimethylthiazol-2-YL]-2,5-diphenyltetrazolium Bromide)
ng	nanogram

OD	Optical density
OKF6	Immortalized human oral keratinocytes
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Pg	Picogram
P.g	*Porphyromonas gingivalis*
PG	Proteoglycan
P.i	*Prevotella intermedia*
RHGE	Reconstructed human gingival epithelium
RHOE	Reconstructed human buccal epithelium
SSC	Side scatter
TIMP	Inhibitors of metalloproteinase
TGF-β	Transforming growth factor-b
TMB	3,3,5,5,-tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
TR146	Human buccal carcinoma keratinocytes
μg	microgram
μl	microliter

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Declaration

I declare that the research design for this thesis was the combined work of my supervisors (Professor Farida Fortune and Dr Alan Cruchley) and the author of this thesis.

All the laboratory work presented in this thesis was carried out by the author with the aid of Dr Alan Cruchley in reconstructed epithelial models. Work in qPCR was in collaboration with Dr Teck Teh. FACS analysis was performed by Dr Gary Warnes. Work in microbiology was in collaboration with Dr Rob Whiley and Dr Ahmed Hashim.
I also declare that this work has not previously been submitted for a higher degree.

Mohammad Hanino, Feb 2011

General Introduction:

Ciclosporin (CsA), a hydrophobic fungal metabolite, is a potent immunosuppressant used as the first choice for counteracting the rejection phenomena in organ transplantation patients and for the treatment of various autoimmune diseases (Condé *et al.,* 2008; Faulds *et al.,* 1993).The use of CsA however is associated with various side effects including neurotoxicity (Chang *et al.,* 2001), hepatotoxicity (Galan *et al.,* 1995), nephrotoxicity (Woolfson & Neild, 1997), hypertension (Textor *et al.,* 1994), and gingival hyperplasia (Seymour *et al.,* 2000).

CsA-induced gingival hyperplasia was first reported by Rateitschak-Plus *et al* in 1983 occurring in 25-81% of patients taking CsA (Seymour & Jacobs, 1992). This lesion usually develops in the first 6 months of CsA administration and initially appears as a papillary enlargement and it is mostly restricted to the keratinized gingiva but it may grow in size with time and cover the crowns of teeth causing difficulties in mastication, speech and profound aesthetic and psychological problems (Marshall & Bartold, 1999; Ilgenli *et al.,* 1999).

Histopathologically, CsA-induced gingival hyperplasia is characterized by an increase in epithelial thickness with elongated rete pegs, increased vascularisation, focal infiltration of plasma cells and to a lesser extent lymphocytes (Buduneli *et al.,* 2001; Mariani *et al.,* 2004), and increased numbers of fibroblasts with accumulation of extracellular matrix components (Mariani *et al.,* 1993; Deliliers *et al.,* 1986).

Despite the intensive studies investigating the cellular and molecular basics of the development of CsA-induced gingival hyperplasia, the exact mechanism underlying this condition is still unclear (Guo *et al.,* 2008). Various risk factors have been described including age, sex, genetic pre-disposition, duration of therapy, concomitant medication such as calcium channel blockers, and gingival inflammation (Bartoli *et al.,* 2004).

However, a recent study suggested that the imbalance between cell proliferation and apoptosis may contribute to the pathogenesis of the hypercellularity observed in CsA-induced gingival hyperplasia (Kantarci *et al.,* 2007).

The effect of CsA on proliferation of HGF is still controversial ranging from absent to an increase or a decrease (James *et al.,* 1995; Bartold, 1989; Pistorius *et al.,* 2003). However, these discrepancies in studies have been attributed to heterogeneity of the HGF strain used (Hassell & Stanek, 1983), or to differences in the experimental conditions applied (Leonardi *et al.,* 2001). Moreover, recent studies suggested that accumulation of gingival fibroblasts observed in drug-associated gingival hyperplasia resulted from the inhibition of apoptosis (Jung *et al.,* 2008; Kantarci *et al.,* 2007).

Furthermore, recent attention has been drawn to the gingival keratinocytes as a potential part of the pathogenesis of CsA-associated gingival hyperplasia. The effect of CsA on gingival keratinocytes is still inconclusive (Cetinkaya *et al.,* 2006) ranging from being pro-proliferative (Lauer *et al.,* 2006), anti-proliferative (Tu *et al.,* 2008), pro-apoptotic (Tu *et al.,* 2009), anti-apoptotic (Buduneli *et al.,* 2007), or no effect (Birraux *et al.,* 2006). However, all of these *in vitro* studies were conducted in monolayer cultures that lack the multilayer structure of the *in vivo* epithelium.

It has been shown that the pathogenesis of CsA-induced gingival hyperplasia is associated with up-regulated level of salivary contents of proinflammatory cytokines including IL-1α, IL-8, and IL-6 compared to healthy controls (Ruhl *et al.,* 2004). Furthermore, it has been suggested that the presence of these cytokines may modulate the cellular response to the drugs (Sato *et al.,* 2005; Sakagami *et al.,* 2006). The effect of CsA on release of IL-6 and IL-8 by HGF *in vitro* was investigated but the results were inconclusive and have been related to the different cell strains used in each study (Maita *et al.,* 2002; Morton & Dongari-Bagtzoglou, 1999). However, there have been no studies on release of these inflammatory cytokines in gingival keratinocytes following treatment with CsA.

The clinical observations of the pathogenesis of CsA-induced gingival hyperplasia indicate a potential role for periodontal bacteria in the pathogenesis. However, most of these studies have been undertaken *in vivo* and the findings are still inconclusive: It is not clear whether accumulation of dental plaque is a consequence of gingival changes caused by the drug itself or it is an essential factor for initiating the pathogenesis (Seymour *et al.,* 2000). This difficulty in determining the exact role of gingival inflammation might be related to the complexity of the *in vivo* situation.

Furthermore, it has been shown that a short course of treatment with Azithromycin, a macrolide antibiotic that acts against both gram-negative and –positive microorganisms, effectively improved the condition of the gingiva in patients with CsA-induced gingival hyperplasia (Condé *et al.,* 2008).

Seymour & Smith (1991) reported that maintaining adequate oral hygiene in humans markedly reduced the severity of CsA-induced gingival hyperplasia but it did not prevent the development of the condition. Animal studies have also shown that dental plaque exacerbated the development of gingival hyperplasia but a marked regression in the condition was noticed when chlorhexidine was applied (Fu *et al.,* 1997; Pilatti *et al.,* 1997). McGaw *et al* (1987) suggested that dental plaque may play a potential role as a local reservoir of CsA that affords a constant deposition of this agent on the juxtaposed surface of gingival epithelium.

Thus, in light of the previous findings it would be valuable to investigate the potential role of pre-existing periodontal status in initiating the pathogenesis of CsA-induced gingival hyperplasia through an *in vitro* study and to investigate whether the exposure to a combination of CsA and bacterial products might modify the cellular response.

Chapter One
Literature Review

1.1 Oral Mucosa:

The mouth is a key point of entry for exogenous material into the body and can be thought of as the gateway to the digestive and the respiratory systems.

The oral mucosa shows structural variation in various regions of the oral cavity and hence it can be categorized into three functional types (Avery *et al.*, 2002):

- Lining mucosa: covers the underside of the tongue, inside of the lips, cheeks, floor of the mouth, and the alveolar processes. This mucosa must be as flexible as possible to perform its function of protection.
- Masticatory mucosa: covers the hard palate and gingiva that are exposed to compressive and shear forces and to abrasion during the mastication of food.
- Specialized mucosa: covers the dorsal surface of the tongue

1.1.1 Functions of the oral mucosa

The oral mucosa has specific functional roles within the oral cavity. The main function is the protection of underlying tissues by resisting mechanical stress (compression, stretching, and shearing), protecting against surface abrasions and also by restricting the invasion by exogenous and endogenous pathogens and materials (Nanci &Ten Cate, 2008).

Another important function of the oral mucosa is its sensory abilities. Receptors within the oral mucosa are able to detect and respond to various stimuli in the mouth (temperature, touch, and pain) as well as initiate reflexes (swallowing, gagging, and salivating) (Nanci &Ten Cate, 2008).

In addition, the oral mucosa maintains the moist surface of the mucous membrane within the oral activity as it contains the ducts of major salivary glands and also is directly associated with all of the minor salivary glands (Nanci &Ten Cate, 2008).

1.1.2. Gingiva:

The gingiva can be defined, in addition to being a part of the periodontium, as an extension of the oral mucosa that covers the alveolar processes and the cervical portions of the teeth (Bartold & Narayanan, 2006; Genco *et al.*, 1990). Anatomically, the gingiva has been divided into three areas: the free marginal gingiva, the

interdental gingiva and the attached gingiva (Bartold *et al.,* 2000). Histologically, the gingival tissue may be divided into two parts:
(a) The gingival epithelium and (b) the gingival connective tissue.

The gingival epithelium:
The gingival epithelium is considered as a first line of defence against both bacterial invasion and mechanical trauma (Dale, 2002). As illustrated in (Fig 1.1), human gingival epithelium is subdivided into three distinct anatomical areas (Nield-Gehrig & Willmann, 2007): (a) oral gingival epithelium (b) sulcular epithelium and (c) junctional epithelium.
(a) Oral gingival epithelium; that lines the outer surface of the gingival tissues. This portion of gingival epithelium is keratinized / parakeratinized stratified squamous epithelium composed of several cell layers (Bartold *et al.,* 2000; Genco *et al.,* 1990; Lindhe *et al.,* 2003): the basal layer (stratum basal or stratum germinativum), stratum spinosum (Prickle cell layer), granular layer, and the cornified layer (stratum corneum).

The cells of the basal layer (often called the stratum germinativum) are either cylindrical or cuboid residing on an uneven basement membrane that separates the epithelium from the connective tissue and protrudes epithelial digital extensions, termed "rete ridges", into the underlying tissue (Bartold *et al.,* 2000; Genco *et al.,* 1990; Lindhe *et al.,* 2003).

Superficial to the basal layer, the cells undergo differentiation resulting in large, polyhedral cells that show short cytoplasmic processes resembling spines and forming the so-called stratum spinosum, the thickest layer of all the epithelial layers (Bartold *et al.,* 2000; Genco *et al.,* 1990; Lindhe *et al.,* 2003). Upward of the stratum spinosum, the cells tend to be more flattened, contain cytoplasmic keratohyaline granules and form the granular layer (Genco *et al.,* 1990; Bartold *et al.,* 2000). In the most superficial layer, the cells become markedly flattened, closely packed and lack their nuclei and organelles transforming into keratinized cells of the stratum corneum (Genco *et al.,*1990; Lindhe *et al.,* 2003; Bartold *et al.,* 2000).

In addition to those keratinocytes, other cell types can also been identified in the gingival epithelium (Bartold *et al.,* 2000; Lindhe *et al.,* 2003) including: Immunocompetent lymphocytes, Langerhans cells, (Bartold *et al.,* 2000; Lindhe *et al.,* 2003) melanin-producing cells**,** Melanocytes, (Bartold *et al.,* 2000; Mayer, 1973) and Merkel cells that are believed to possess a sensory role (Lindhe *et al.,* 2003).

(b) Sulcular epithelium: the epithelial tissue that lines the gingival sulcus.

(c) Junctional epithelium: the epithelium tissue that forms the bottom of the gingival sulcus and attaches the gingiva to the tooth surface.

Both the oral sulcular and the junctional epithelium are non-keratinized, stratified squamous epithelium with a very fast cellular turnover rate (Gorrel *et al.,* 2004).

However, lacking the cornified cell layer in both the sulcular and the junctional epithelium makes them a less effective protective barrier. Consequently, the sulcular and junctional areas of the oral epithelium are regarded as weak points for entry into and invasion of the gingival connective tissue by bacteria and bacterial products (Nield-Gehrig & Willmann, 2007).

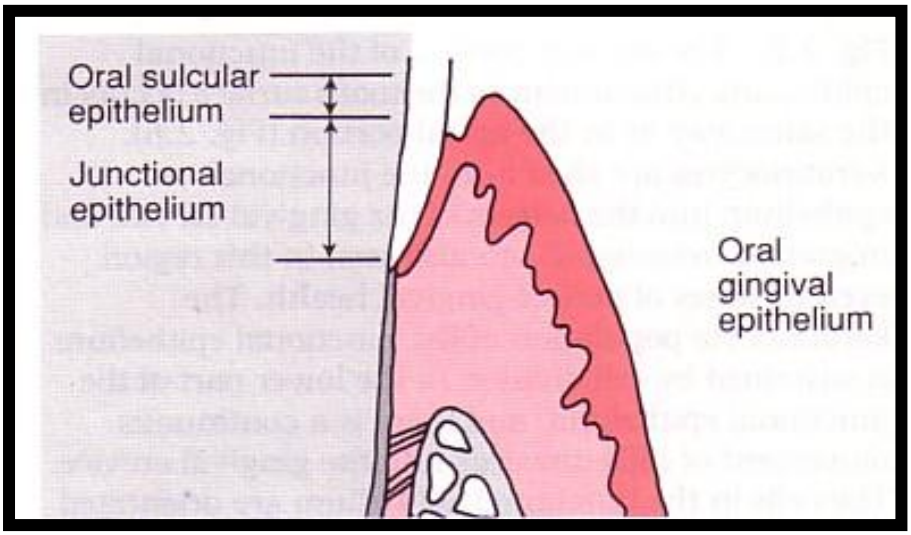

Figure 1.1. Microscopic histological anatomy of the gingival epithelium junctional epithelium, oral sulcular epithelium, and oral gingival epithelium. (Taken from Williams, Hughes, Odell & Farthing. Pathology of Periodontal Disease. Oxford Medical Publications ISBN 0192619551 1992).

The gingival connective tissue:

The gingival connective tissue consists primarily of cells, extracellular matrix, nerves and blood vessels (Genco *et al.,* 1990). The predominant cell in the gingival connective tissue is the fibroblast which has been considered as the engineer and architect of the extracellular matrix by maintaining an organized synthesis and turnover of collagen and glycosaminoglycans (Morton & Bagtzoglou, 1999; Chae *et al.,* 2006). Furthermore, gingival fibroblasts possess the ability to express a variety of cytokines including IL-1, IL-6, and IL-8 (Modéer *et al.,* 2000). Additionally, other less common cells may normally be identified in the gingival connective tissue including undifferentiated mesenchymal cells, mast cells and macrophages (Genco *et al.,* 1990; Lindhe *et al.,* 2003).

Extracellular matrix (ECM):

Extracellular matrix represents the material that surrounds all connective tissue cells and underlies all epithelia and endothelia (Embery *et al.,* 2000) deriving its importance not only from being a supporting system of tissue's physical structure, but also from its crucial role in regulating proliferation, development, migration and function of cells (Embery *et al.,* 2000; Martinez & Araújo, 2004).

The extracellular matrix of the gingival connective tissue consists of collagens and non-collagenous proteins (Vardar *et al.,* 2005; Embery *et al.,* 2000). Collagens comprise approximately three fifth (60%) of the total proteins present (Mariotti, 1993; Palaiologou *et al.,* 2001) and five types of collagen have been identified throughout the gingival connective tissue including types 1, 3, 4, 5 and 6 (Mariotti, 1993; Narayanan *et al.,* 1980).

With respect to the non-collagenous proteins, they are primarily composed of core protein of proteoglycans (PGs) covalently attached to one or more highly anionic glycosaminoglycan (GAG) chains (Embery *et al.,* 2000; Vardar *et al.,* 2005). Of the glycosaminoglycans currently recognized dermatan sulphate, chondroitin-4-sulfate, heparin sulphate and hyaluronic acid are the most abundant (Embery *et al.,* 2000).

Immunohistochemical studies have also shown a number of proteoglycans in the underlying connective tissue including decorin, biglycan, versican and syndecan

(Embery *et al.*, 2000), in addition to a variety of adhesive glycoproteins including fibronectin, tenascin, laminin, and other glycoproteins such as osteonectin (Embery *et al.*, 2000; Mariotti, 1993).

1.2. Gingival Hyperplasia

1.2.1. Hereditary gingival fibromatosis, also termed idiopathic gingival overgrowth, hereditary gingival hyperplasia, and elephantiasis gingivae (Coletta & Graner, 2006; Häkkinen & Csiszar, 2007), is a rare genetic disorder characterized by spontaneously and slowly progressive gingival enlargement. It can be generalized affecting both the maxilla and mandible or localized in specific areas involving, in particular, the maxillary tuberosities and the labial gingiva around the lower molars (Coletta & Graner, 2006; Häkkinen & Csiszar, 2007). The appearance of gingival enlargement usually initiates at the time of permanent tooth eruption and, in fewer cases, at the time of eruption of the primary teeth, but it is rarely present at birth (Häkkinen & Csiszar, 2007). Furthermore, the development of this condition appears to be correlated to the presence of teeth since disappearance of this lesion in edentulous patients has been reported in many studies (Coletta & Graner, 2006; Häkkinen & Csiszar, 2007).

Hereditary gingival hyperplasia can be identified as an isolated disease involving only the gingivae or as a manifestation of a variety of syndromes (such as: Zimmerman-Laband syndrome, Cross syndrome, Rutherford syndrome, Ramon syndrome, Juvenile hyaline fibromatosis, and Jones syndrome) in association with other characteristics including hypertrichosis, mental retardation, epilepsy, hearing loss, supernumerary teeth, and abnormalities of the fingers and toes (Häkkinen & Csiszar, 2007; Clocheret *et al.*, 2003).

1.2.2. Drug-induced gingival hyperplasia:

Drug-induced gingival hyperplasia is a common side effect associated with the systemic administration of several drugs (Güncü *et al.*, 2006). These drugs can be categorized into three main classes:

Anticonvulsants: such as phenytoin, the first choice for controlling the convulsive seizure disorders in patients experiencing epilepsy (Kato *et al.*, 2005; Hallmon &

Rossmann, 1999). The development of phenytoin-induced gingival hyperplasia was first reported in 1939 by Kimball and is estimated to occur in up to 57% of patients (Brunet *et al.,* 2001).

Calcium channel blockers:, also known as calcium antagonist (Hallmon & Rossmann, 1999) including nifedipine, diltiazem and verapamil, are antihypertensive agents widely used in elderly individuals experiencing angina and various cardiovascular diseases (Nishimura *et al.,* 2002). The use of these therapeutic agents has also been associated with the development of gingival hyperplasia in approximately 10% of patients (Seymour, 2006).

CsA: an immunosuppressive agent broadly used to counteract organ transplant rejection phenomenon and to treat various autoimmune disorders such as bullous pemphigoid, psoriasis and rheumatoid arthritis (Bostrom *et al.,* 2005). CsA-induced gingival hyperplasia was first described in 1983 by Rateitschak-Plüss (Reviewed by Das *et al.,* 2002) and is now estimated to occur in up to 30% of CsA-treated patients (Seymour, 2006).

1.2.3. Clinical Features of Drug-induced gingival Hyperplasia:

Gingival hyperplasia usually begins within 1-3 months of drug administration as a papillary enlargement that increases in size with continuing treatment, reaching its typical marked lobulated nodular appearance after 12- 18 months (Kuru *et al.,* 2004; Tyldesley *et al.,* 1984). The condition is usually more prominent in the facial aspects of the anterior segments (Seymour & Jacobs, 1992; Strachan *et al.,* 2003; Wilson *et al.,* 1998) but it may also be present throughout the mouth (Thomas *et al.,* 1991). The clinical presentations of gingival hyperplasia induced by CsA, nifedipine or phenytoin are largely similar (Thomas *et al.,* 1991; James *et al.,* 2000; Brunet *et al.,* 2001; Akimoto *et al.,* 1991) ranging from being firm and fibrous in consistency to spongy and oedematous. It may also be hemorrhagic with secondary inflammation. (Daley & Wysocki, 1984; James & Linden, 1992; Eslami *et al.,* 2004).

Gingival hyperplasia is often localised to the attached gingiva (Marshall & Bartold, 1999), but in severe cases it may extend lingually, facially, and coronally partially or completely involving the crowns. These changes may cause difficulty with

mastication and speech, halitosis, tooth displacement, delayed eruption and psychological stress due to the aesthetic appearance (Strachan *et al.*, 2003; Kato *et al.*, 2005) (Fig 1.2).

As a result of these different clinical presentations, gingival hyperplasia has been graded into four categories (Gómez *et al.*, 1997; Miranda *et al.*, 2005) according to criteria of Angelopoulos & Goaz (1972) modified later by Miller & Damm (1992) as following:

Score 0: no gingival hyperplasia (normal gingiva)

Score 1: mild gingival hyperplasia, in which the hyperplastic gingiva (as much as 2mm or less) covers only the cervical third or less of the tooth crown.

Score 2: moderate gingival hyperplasia, where the hyperplastic gingiva (ranging Between 2 to 4 mm) extends to the middle third of the tooth crown.

Score 3: severe gingival hyperplasia, in which the overgrown gingiva (more than 4mm) covers more than two thirds of the tooth crown.

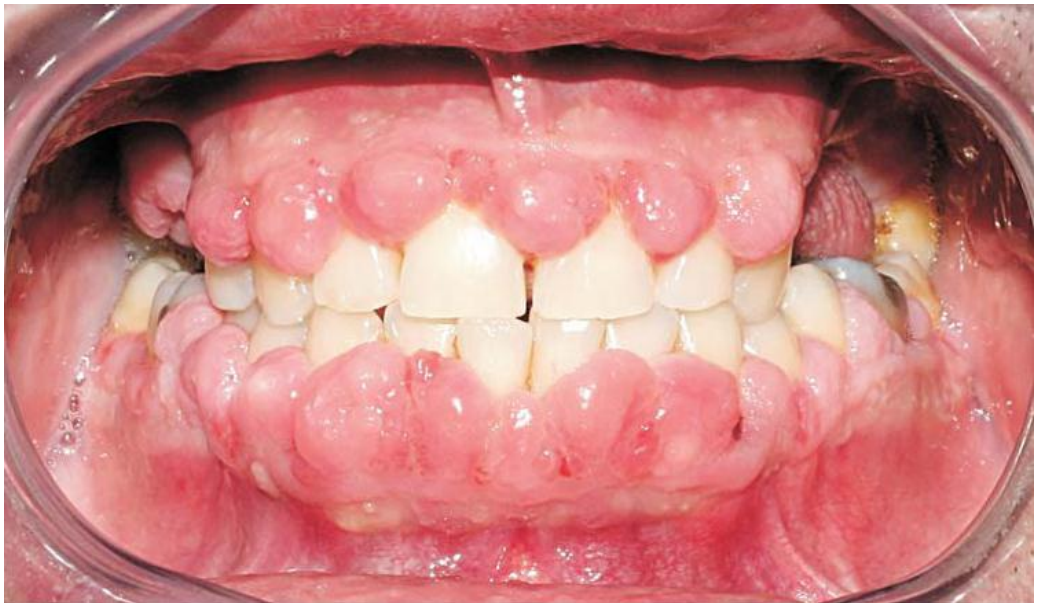

Figure 1.2 A typical clinical status of CsA-induced gingival hyperplasia in 45 year old kidney transplant male patient after 10 months of treatment with CsA. (Taken from (Popova & Mlachkova, 2007).

1.2.4. Histopathological Features of drug-induced gingival Hyperplasia:

The microscopic examination of sections of drug-induced hyperplastic gingival tissue shows increased thickening of the epithelium associated with different degree of

acanthosis and parakeratosis and elongated rete pegs (Collan *et al.,* 1982; O'Valle *et al.,* 1994; Spolidorio *et al.,* 2004; Mariani *et al.,* 2004; Hyland *et al.,* 2004; Pisanty *et al.,* 1988; Pisanty *et al.,* 1990). Enlargement of the underlying gingival connective tissue may also be present. This may be the result of increased vascularity, accumulation of the extracellular matrix materials involving amorphous ground substance and irregular collagen bundles (mostly Collagen type 1 and 3) and a significant increase in elongated fibroblasts (Mariani *et al.,* 1993; Ayanoglou & Lesty, 1999). Focal infiltration of inflammatory cells, mainly plasma cells and to a less degree lymphocytes were also reported (Buduneli *et al.,* 2001).

1.2.5. Prevalence of drug-induced gingival hyperplasia:

Three main categories of drug –induced gingival hyperplasia (CsA, calcium channel blockers, and phenytoin) have been recognized but so far there is no agreement with respect to an accurate determination of the prevalence rate in each drug category (Costa *et al.,* 2007; Seymour, 2006). Some studies report a wide range of prevalence rate estimated between 10% to 50% for phenytoin, 8% to 70% for CsA, and 0.5% to 83% for nifedipine (Kataoka *et al.,* 2005; Nishikawa *et al.,* 1996; Seymour *et al.,* 1988; Barak *et al.,* 1987). Other reports however, have shown a prevalence rate of 50% for phenytoin, with much lower prevalence for CsA and calcium channel blockers which are 30% and 10% respectively (Seymour, 2006; Seymour *et al.,* 2000). This great variability in the prevalence rate may stem from differences in clinical criteria and methods used in diagnosis and measurement of gingival changes, the characteristics and number of subjects involved in the study sample, in addition to other factors including the concomitant use of other drugs which might influence the condition, age, gender, and drug variables (Romito *et al.,* 2004; Afonso *et al.,* 2003; Brunet *et al.,* 2001; Costa *et al.,* 2006).

1.2.6. Risk Factor for drug-induced gingival hyperplasia:

1.2.6.1. Demographic variables:

Age:

Age has been identified as a significant predisposing factor for the development of gingival hyperplasia in patients treated by CsA or phenytoin (Seymour, 2006; Daley *et al.,* 1986; Esterberg, 1945; Hefti *et al.,* 1994) or those taking a combination of CsA and nifedipine (Thomason *et al.,* 1997). Age has not been reported as a relevant risk

factor for calcium channel blockers-induced gingival hyperplasia since these drugs have narrow spectrum of efficiency restricted mainly to management of certain situations such as hypertension which are more common in the post middle-aged (Kataoka *et al.,* 2005; Seymour *et al.,* 2000). The increased susceptibility to phenytoin or CsA -induced gingival hyperplasia observed in adolescents could be ascribed to the high sensitivity of young gingival fibroblasts to the challenging medication (Wright *et al.,* 2005; Daly *et al.,* 1992).

Gender:

Most available data point out that CsA-induced gingival hyperplasia has a higher incidence and severity in men (Thomason *et al.,* 1995; Thomason *et al.,* 1996; Tyldesley & Rotter, 1984). Furthermore, some studies have shown that males receiving calcium channel blockers were three times more likely than females to be affected by gingival enlargement (Ellis *et al.,* 1999). In contrast, other reports have revealed no such a positive correlation between gender and development of gingival hyperplasia in patients treated by phenytoin (Güncü *et al.,* 2006; Brunet *et al.,* 2001) or with CsA alone or a combination of CsA and nifedipine (Somacarrera *et al.,* 1994; Pernu *et al.,* 1992; Cebeci *et al.,* 1996; King *et al.,* 1993).

1.2.6.2. Pharmacokinetic Variables:

Drug Formulation:

Wondimu *et al* (1996) reported that patients treated with CsA solutions had earlier onset and more severe gingival hyperplasia than those receiving CsA capsules. These findings may be ascribed either to differences in the drug's pharmacokinetics in terms of bioavailability and time to peak blood concentrations, or to a potential local impact of CsA achieved by the higher salivary CsA levels available in individuals taking CsA solutions than those ingesting CsA capsules (Seymour *et al.,* 2000; Modeer *et al.,* 1992).

Duration of therapy:

Although most studies have shown no positive correlation between the duration of drug administration and progress of gingival hyperplasia (Pernu *et al.,* 1992; King *et al.,* 1993; Pernu *et al.,* 1993; Wondimu *et al.,* 1993; Vescovi *et al.,* 1997; Thomas *et*

al., 2001), other reports have demonstrated that sharp onset in development of gingival hyperplasia occurs within the first 3-6 months and then steadily progress (Daley *et al.,* 1986; Somacarrera *et al.,* 1994). These findings were supported by animal studies that have shown a positive relationship between gingival hyperplasia induced by CsA, nifedipine, and phenytoin and the duration of therapy (Khoori *et al.,* 2003; Nishikawa *et al.,* 1996).

Drug dosage and drug serum concentration:

The role of drug dosage and drug serum concentration in the development of gingival hyperplasia has remained a controversial issue ranging from findings presenting a strong association between the progression of gingival hyperplasia and both dose and drug serum levels (Thomas *et al.,* 2000; Costa *et al.,* 2007; Addy *et al.,* 1983; Thomason *et al.,* 1997), to contrasting reports showing no statistically significant link (Büchler *et al.,* 2004; Brunet *et al.,* 2001; Nery *et al.,* 1995). There is, however, a general consensus that a threshold concentration of the drug may be necessary to elicit the gingival alterations, taking into account the potential variation of the threshold concentration either inter-individually or from one drug to another (Seymour, 2006; Chabria *et al.,* 2003; Somacarrera *et al.,* 1994).

Salivary drug concentration:

Conflicting data similar to those observed with the drug serum concentration parameter have also been reported for the salivary drug concentration index (Seymour *et al.,* 2000).

1.2.6.3. Genetic Factors:

Gingival fibroblast Heterogeneity:

Some authors have attributed the inter-individual variation in the susceptibility to gingival hyperplasia- inducing drugs to phenotypic and functional heterogeneity among subpopulations of gingival fibroblasts in relation to proliferation, synthesis of extracellular matrix, expression of collagenase enzymes, tissue inhibitor of metalloproteinase activity and response to challenging drugs (Chabria *et al.,* 2003; Myrillas *et al.,* 1999; Doufexi *et al.,* 2005). In light of this fact, patients who develop drug-induced gingival hyperplasia may have drug-sensitive gingival fibroblast strains versus drug-resistant gingival fibroblast subpopulations that could be the most

predominant type in those non-responders to drugs (Flynn *et al.*, 2006; Varga *et al.*, 1998).

Human leukocyte antigen (HLA) Expression:

Many studies have concentrated on the potential role of human leukocyte antigens (HLA) phenotype as a risk factor for drug-induced gingival hyperplasia (Wright *et al.*, 2005, Vescovi *et al.*, 2005). However, most findings concur that individuals expressing HLA-DR2, A24, and B37 may be more susceptible to developing this side effect than those who express HLA-DR1 (Seymour, 2006; Thomason *et al.*, 1996; Seymour *et al.*, 2000).

Cytochrome P450 enzyme polymorphism:

Hepatic cytochrome P450 enzymes are primarily responsible for the metabolism of phenytoin, CsA, and nifedipine, and some studies have suggested that P450 polymorphism may explain the variation in incidence and severity of drug-induced gingival hyperplasia among patients (Seymour *et al.*, 2000; Doufexi *et al.*, 2005).

α2 integrin polymorphism:

α2 integrin is a transmembrane glycoprotein receptor mediating the initial step of collagen phagocytosis by promoting fibroblast adhesion to type I collagen (Kataoka *et al.*, 2003; Ogino *et al.*, 2005). Recent investigations have revealed that the decreased levels of α2 integrin reported in patients with drug-induced gingival hyperplasia were more frequently associated with specific gene (α2 integrin 807 C allele) than that in healthy control (Ogino *et al.*, 2005).

Cytokine gene polymorphism:

Despite the putative pivotal role of cytokines in the pathogenesis of drug-induced gingival hyperplasia (section 1), little is known about the influence of cytokine gene polymorphisms on development of the condition. However, while some studies have failed to report any positive correlation between IL-6 gene polymorphism and incidence or severity of CsA-induced gingival overgrowth (Drozdzik *et al.*, 2005), a recent report has shown that IL-1α -889 alleles were considerably more frequent in healthy control than those experiencing CsA- induced gingival enlargement. Therefore, these findings have suggested that IL-1α gene polymorphism might be

responsible for the variable susceptibility to CsA in terms of gingival hyperplasia development (Bostanci *et al.*, 2006).

Transforming growth factor (TGF-β1) gene polymorphism:

In agreement with findings that have revealed that TGF-β1 levels either in plasma (at low concentrations) or in GCF (at higher levels) might be indicators for progress of gingival hyperplasia in patients concomitantly treated by CsA and nifedipine (Wright *et al.*, 2004; Ellis *et al.*, 2004), genetic investigations have also shown a positive correlation between TGF-β1 gene polymorphism and expression of gingival hyperplasia induced by concomitant use of CsA and nifedipine (Linden *et al.*, 2001).

Multidrug resistance (MDR1) gene polymorphism:

MDR1 genes encode the drug transporter P-glycoprotein (P-gp) which is important for the absorption, distribution and elimination of gingival hyperplasia- inducing drugs. Hence, any variation in P-gp expression will lead to pharmacokinetic variables of the drug, consequently, discrepancy in the effect of the drug (Wei *et al.*, 2005; Meisel *et al.*, 2006). While some studies have found no evidence of a relationship between the MDR1 gene polymorphism and development of CsA-induced gingival hyperplasia (Drozdzik *et al.*, 2004), other reports have shown a marked association between this specific gene polymorphism and calcium channel blockers-induced gingival enlargement (Meisel *et al.*, 2006).

1.2.6.4. Concomitant medication:

The effect of concomitant medication on variability of incidence and severity of drug-induced gingival hyperplasia has been extensively studied (Seymour, 2006; Wilson *et al.*, 1998). A decreased severity of gingival hyperplasia has been reported in patients concurrently receiving CsA and other immunosuppressant such as azathioprine and prednisolone (Seymour *et al.*, 2000; Hassell & Hefti, 1991; Somacarrera *et al.*, 1994) that might be ascribed either to the well-known anti-inflammatory efficacy of azathioprine and prednisolone against dental plaque (Thomason *et al.*, 2005) or to the lower CsA dosages used on this multiple immunosuppressive regimen (Costa *et al.*, 2006; Costa *et al.*, 2007; Thomason *et al.*, 2005).

Other studies have reported that patients treated with CsA in conjunction with calcium channel blockers have shown a higher incidence of gingival enlargement than those maintained on CsA alone (Seymour *et al.,* 2000; Bokenkamp *et al.,*1994; O'valle *et al.,* 1995). These findings have been attributed to the synergistic impact of the drug combination on the gingival tissue (Spolidorio *et al.,* 2003; Chabria *et al.,* 2003).

The use of phenobarbital and/or carbamazepine along with phenytoin has also been markedly associated with higher prevalence of gingival overgrowth than that with phenytoin alone (Seymour, 2006; Brunet *et al.,* 2001).

1.2.6.5. Periodontal Variables:

There is a wealth of conflicting data concerning the exact role of dental plaque and gingival inflammation in the aetiology of CsA-induced gingival hyperplasia ranging from whether it is a contributory factor or merely a consequence of the gingival enlargement (Afonso *et al.,* 2003; Seymour *et al.,* 2000). Some studies have shown that dental plaque has no essential role in the development of gingival hyperplasia induced by CsA alone or by combined administration of CsA and nifedipine, and also claimed that plaque control has only limited efficacy in controlling this disease (Khoori *et al.,* 2003; Bostanci *et al.,* 2006; Friskopp & Klintmalm, 1986). Other reports, however, have recognized the plaque index and gingivitis as considerable risk factors for the expression of gingival overgrowth (Aimetti *et al.,* 2005; Somacarrera *et al.,* 1994). These postulations are based on the potential role of dental plaque as a reservoir for CsA which affords affective and continuous local concentrations of CsA (Niimi *et al.,* 1990) and were supported by other findings that have shown that the local bioavailability of CsA is much higher in inflamed sites than in non-inflamed ones (Thomas *et al.,* 2000).

There has been strong evidence supporting the importance of the plaque index in phenytoin-induced gingival hyperplasia in which the enhanced accumulation of collagen in response to phenytoin appears to be promoted by dental plaque-induced chronic gingivitis (Akiyama *et al.,* 2006). Furthermore, the importance of bacterial gingivitis in emerging gingival hyperplasia induced by nifedipine has also been reported by other studies (Seymour *et al.,* 2000; Eslami *et al.,* 2004) These findings

were based on the clinical observations that did not show any sign of enlargement in edentulous regions (Eslami *et al.,* 2004).

However, despite the conflicting information on the importance of dental plaque in this pathogenesis, there is a consensus that bacterial gingivitis at least exacerbates the emerging of CsA-induced gingival hyperplasia and that good oral hygiene improves the condition (Seymour *et al.,* 2000; Seymour, 2006).

1.3 Oral Bacteria:

The oral cavity is a habitat for over than 300 species of microorganisms that colonize the oral and gingival mucosa (Han & Shi, 2000). These bacteria can be classified as commensal (non-pathogenic) and pathogenic bacteria (Kimball *et al.,* 2006). Among the most predominant periodontopathic bacteria are the anaerobic Gram-negative bacteria including *Porphyromonas gingivalis* (P.g), *Aggregatibacter actinomycetemcomitans* (A.a), *Prevotella intermedia* (P.i), *Fusobacterium nucleatum* (F.n), and *Tannerella forsythia* (T.f) in addition to oral spirochetes such as *Treponema denticola* (T.d) (Han & Shi, 2000; Bascones & Figuero, 2005; Sela, 2001). The periodontopathic bacteria secrete a variety of extracellular virulence factors that initiate and progress the periodontal diseases by affecting various pathways including cytokine expression, cell cycle and apoptosis (Huang *et al.,* 2001; Sugai & Kawamoto, 1998; Stathopoulou & Galicia, 2009)

The relationship between periodontal bacteria and CsA-induced gingival hyperplasia:
Increasing attention has been focused on the role of periodontal bacteria in the initiation and progression of gingival hyperplasia in patients taking CsA but this relationship has yet to be clearly defined. Clinical observations, however, revealed that development of gingival hyperplasia in patients taking CsA is highly related to the oral hygiene status (Vescovi *et al.,* 2005). In addition, McGaw *et al* (1987) indicated the potential role of dental plaque as a local reservoir of CsA that may afford a constant deposition of this drug on the adjacent gingival epithelium.

Gong *et al* (2008) found that *P. gingivalis, T. forsythia* and *T. denticola* were more frequently present in patients taking CsA and developing gingival hyperplasia

compared to those patients who were taking CsA but did not develop the condition. These observations were in agreement with Leung *et al'*s report (2003) that found that gram-negative rods and spirochetes were the main bacterial species being detected in the subgingival microflora from patients taking CsA and developing chronic periodontitis compared to CsA-treated patients without periodontitis.

Moreover, Romito *et al* (2004) reported that microorganisms including A. actinomycetemcomitans (23%), *P. gingivalis* (36%), *P. intermedia* (93%), *Fusobacterium sp*. (66%), *Campylobacter rectus* (30%), *Micromonas micros* (66%), enteric rods (0%), and yeasts (30%) were isolated from patients taking CsA with or without gingival hyperplasia. *Micromonas micros,* however, was more frequently present in patients with gingival hyperplasia indicating that colonization of oral cavity by this microorganism might play a role in the pathogenesis. Furthermore, these clinical findings were supported by an animal study that found that the severity of inflammation and the subsequent development of gingival hyperplasia were enhanced by the existence of large amounts of gram negative rods and anaerobic bacteria in CsA- treated ferret and subject to experimental ligature periodontitis (Fischer *et al.,* 1996).

These findings were in parallel with other microbiological studies that were carried out in patients developing gingival hyperplasia induced by phenytoin and nifedipine. Akiyama *et al* (2005) reported that *P. gingivalis* and *T. denticola* had a positive correlation with the development of gingival hyperplasia in patients taking phenytoin. Takada *et al* (2003) also found that black pigmented obligate anaerobic Gram-negative rods, particularly, *P. intermedia* and to a less degree *P. gingivalis* were predominately existed in the subgingival microflora of patients developing phenytoin-induced gingival hyperplasia. Nakou *et al* (1998) found that *F. nucleatum* was one of the most prevalent subgingival bacterial species being isolated from the nifedipine-associated gingival enlargement lesions in human.

In contrast with these findings, Romito *et al* (2003) did not find a positive correlation between Gram-negative enteric rods and development of gingival hyperplasia in patients taking CsA. However, Saraiva *et al* (2006) found that transplant surgery patients who were under a CsA regimen showed a quantitative and qualitative

modulation in their subgingival microflora after 90 days of CsA administration but there was no positive correlation between development of gingival hyperplasia and a specific species of bacteria.

Overall there seems to be a general consensus that anaerobic Gram-negative bacteria and oral spirochetes predominate in the subgingival microflora in patients experiencing CsA-induced gingival hyperplasia. However, it is not clear if the change in periodontal microflora is due to the immunosuppressive action being exerted by CsA, a subsequent result of gingival hyperplasia or whether the patient's periodontal status may play a role in pathogenesis of CsA-induced gingival hyperplasia.

1.4. Ciclosporin:

1.4.1. History, uses and effects:

CsA is a fermented fungal metabolite of Tolypocladium inflatum W. Gams that has a lipophilic cyclic polypeptide structure composed of 11 amino acids (Moussaïf *et al.,* 1997; Lauer *et al.,* 2006). It was discovered accidentally in the early 1970s by Jean Borel (reviewed-Thomas *et al.,* 1991; Dreyfuss *et al.,* 1976). It is a potent immunosuppressive agent (Moussaïf *et al.,* 1997), and since its introduction, has become the first choice for counteracting the rejection phenomenon following human organ transplantation (Guaguere *et al.,* 2004; Calne *et al.,* 1978; Thomason *et al.,* 2005; Kahan, 1989). It had improved the 5-year survival rate following organ transplantation from 50% to 90% (Thomas *et al.,* 1991) and also improved the management of some autoimmune diseases (Hassell & Hefti, 1991; Faulds *et al.,* 1993) such as psoriasis (Ellis *et al.,* 1986), atopic dermatitis (Hoare *et al.,* 2000), uveitis (Duarte *et al.,* 2003), rheumatoid arthritis (Cutolo *et al.,* 1997), pemphigus (Thivolet *et al.,* 1985), type1 diabetes mellitus (Khoori *et al.,* 2003), Behcet's disease (Koh *et al.,* 2004), lupus erythematosis (Isenberg *et al.,* 1980), myasthenia gravis (Morton *et al.,* 1999), Crohn's Disease (Faulds *et al.,* 1993), multiple sclerosis and primary biliary cirrhosis (Wright *et al.,* 2005), immune-mediated ocular diseases such as Sjögren syndrome, vernal keratoconjunctivitis and corneal ulcers (Leonardi *et al.,* 2001).

There are, however, several adverse side effects associated with the use of CsA including nephrotoxicity (Woolfson *et al.,* 1997), hepatotoxicity (Galan *et al.,* 1995;

Klintmalm *et al.,* 1981), neurotoxicity (Beaman *et al.,* 1985; Chang *et al.,* 2001), hypertension (Arzate *et al.,* 2005), hirsutism (Laupacis *et al.,* 1982; Vescovi *et al.,* 2005), diabetes (Thomason *et al.,* 2005), osteoporosis (Cueto-Manzano *et al.,* 1999; Guo *et al.,* 1998), cutaneous disorders (Afonso *et al.,* 2003; Menni *et al.,* 1991) and opportunistic fungal and viral infections (Seymour *et al.,* 1997).

In addition, CsA use may also be accompanied by oral complications including: gingival hyperplasia (O'Valle *et al.,* 1994; Seymour *et al.,* 1997; Linden *et al.,* 2001; Dongari *et al.,* 1993), Kaposi's sarcoma (Stabellini *et al.,* 2004), lingual fungiform papillae hypertrophy (Marshall *et al.,* 1999), hairy leukoplakia and increased disposition to malignant transformations such as lip cancer (Wright *et al.,* 2005). CsA also appears to affect dento-alveolar bone mineralization with the deposition of irregular cementum, decreased bone volume and increased marrow volume (Ayanoglou *et al.,* 1997; Chin Shen *et al.,* 2001).

1.4.2. Ciclosporin Pharmacokinetics:

CsA is absorbed primarily in the upper small intestine (Wright *et al.,* 2005) and peak CsA concentrations in plasma are attained at about 3-4 hours (Hallmon & Rossmann, 1999). This absorption, however, depends largely on bile secretion [which is required for emulsification of the formulation (Guaguère *et al.,* 2004; Steffan *et al.,* 2004; Venkataramanan *et al.,* 1986; Takaya *et al.,* 1989)], oil droplet size, gastrointestinal motility, gastric emptying, and any concurrent medication (Wright *et al.,* 2005; Katz 1997; Steffan *et al.,* 2004). Thus, the variation in these factors could explain the great inter-individual variability in terms of pharmacokinetic aspects of CsA (Guaguère *et al.,* 2004).

CsA is highly bound to blood cells and plasma components. Hence, it readily distributes in various tissues (Mendonza *et al.,* 2004) and it is effectively metabolized in the liver and to a less degree in the intestines by the cytochrome P450 enzymes CYP3 A4 where the CsA molecule undergoes hydroxylation and demethylation yielding multiple metabolites (Steffan *et al.,* 2004, Maurer *et al.,* 1984, Vickers *et al.,* 1992, Whalen *et al.,* 1999). Finally, CsA which has a serum half-life ranging from 17 to 40 hours (Hallmon & Rossmann, 1999) is excreted primarily via the bile through

faeces with less than 10% eliminated through the kidneys as unchanged CsA (Venkataramanan *et al.,* 1988; Guaguère *et al.,* 2004).

1.4.3. Mechanism of the immunosuppressive action of Ciclosporin:

CsA exerts its specific immunosuppressant activity by directly targeting the T-lymphocytes (Stabellini *et al.,* 2004) by binding to its respective cytoplasmic receptors called cyclophillins, a group of the immunophillin family receptors available abundantly in T-cells (Handschumacher *et al.,* 1984; Schreiber, 1991). The CsA- cyclophillin complex then interacts with and inhibits calcineurin, a calcium/calmodulin-dependent serine/threonine phosphatase (Mendonza *et al.,* 2004, Büchler *et al.,* 2004). Calcineurin plays a pivotal role in the transcriptional activation of mRNA encoding IL-2 and other cytokines signalling by calcineurin pathways such as IL-4 and interferon (IFN)-γ (Guaguere *et al.,* 2004, Rao *et al.,* 1997). Blocking of dephosphorylation results in suppression of IL-2 expression (Almawi *et al.,* 2000) thereby impeding T-cell activation (Fierro *et al.,* 2003).

CsA also targets and inhibits other cells involved in the immune response including both macrophages and B- lymphocytes (Yagiela *et al.,* 2004; Cutolo *et al.,* 1997).

1.5. Mechanism of CsA-induced gingival hyperplasia:

The mechanism underlying the development of CsA-induced gingival hyperplasia is still unclear. But it has been suggested that this pathogenesis might reflect complex interactions between the drug, gingival tissue and local released mediators (Aimetti *et al.,* 2008; Morton & Bagtzoglou, 1999) (Fig 1.3).

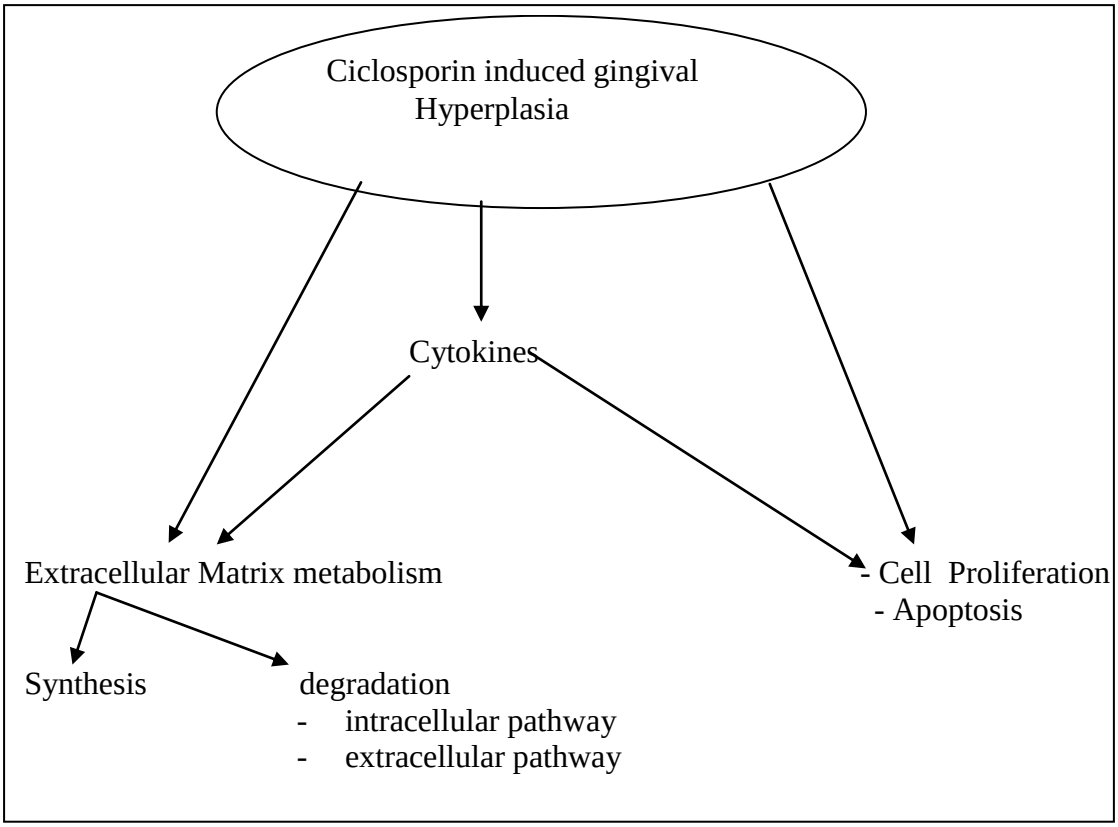

Figure 1.3. Possible pathways of CsA-induced gingival hyperplasia

1.5.1. Effect of CsA on extracellular matrix (ECM) metabolism:

The physiological balance of ECM metabolism is maintained by equilibrium between collagen synthesis and degradation (Lucattelli *et al.,* 2003; Laurent, 1987; Bartold, 1995). In the gingival tissue, disrupted homeostasis between breakdown and expression of ECM may lead either to excessive ECM accumulation (fibrosis) or to ECM destruction (periodontitis) (Tüter *et al.,* 2002; Woessner, 1994; Chong *et al.,* 2007).

1.5.1.1. Effect of CsA on ECM synthesis:

The mechanism whereby CsA induces excessive accumulation of the gingival connective ECM is still a controversial issue ranging from up-regulation of ECM synthesis either at gene transcription or protein level (Gagliano *et al.,* 2004; Schincaglia *et al.,* 1992) to blocking the proteolytic cascade of matrix proteins (Gagliano *et al.,* 2004), or both (Tüter *et al.,* 2002). However, recent findings have

revealed that the increased deposition of collagen 1 observed in CsA-induced gingival hyperplasia is caused by up regulation of both collagen 1 protein and gene expression along with increased deposition of decorin- a proteoglycan known for its inhibitory effects of collagen 1 internalization, thus impeding collagen phagocytosis (Dannewitz *et al.,* 2006).

Other studies have shown that CsA-induced gingival hyperplasia has been associated with increased expression of chondroitin-4-sulphate (C4S) rather than proteoglycans (PGs) (Vardar *et al.,* 2005). This result may provide additional support for an interesting explanation which describes the dimensional changes in CsA-induced gingival hyperplasia as tissue oedema resulting from increased trapping of fluids in the ECM, thus an increase in the tissue colloid osmotic pressure due to the excessive accumulation of GAGs in the ground substance (Vardar *et al.,* 2005; Zebrowski *et al.,* 1994; Flynn *et al.,* 2006; Stabellini *et al.,* 2004). These findings have been sustained by an *in vitro* study concluding that fibroblast cultures isolated from CsA-hyperplastic gingiva show increased synthesis of GAGs in comparison with control normal fibroblasts (Stabellini *et al.,* 2004). In contrast, (Rocha *et al.,* 2000) found no significant difference in the synthetic activity of GAGs between CsA-stimulated gingival fibroblasts and control healthy fibroblasts.

1.5.1.2. Effect of CsA on ECM degradation:

Two primary pathways of ECM breakdown have been identified (Lee *et al.,* 2006; Sodek & Overall, 1988; Everts *et al.,* 1996):

1-Extracellular pathway: involving metalloproteinases (MMPs, plasmin and stromelysin and tissue inhibitors of metalloproteinases (TIMPs)

2- Intracellular pathway: This route involves phagocytosis and digestion of collagen fibrils by fibroblasts's lysosomal cysteine proteinases (cathepsins).

Effect of CsA on Extracellular Pathway of ECM degradation:

Many investigations have traced the variations in activity and expression of both proteolytic enzymes (MMPs) and their specific tissue regulators (TIMPs) that may occur during CsA therapy (Gagliano *et al.,* 2004; Yamada *et al.,* 2000; Bolzani *et al.,* 2000; Hyland *et al.,* 2003).

Hyland *et al* (2003) revealed that CsA down-regulated both MMP-1 gene and protein production in gingival fibroblasts at a concentration of 500-2000ng/ml. Bolzani *et al* (2000) also demonstrated that CsA inhibited MMP-2's gelatinolytic activity in both CsA-treated human gingival fibroblasts and CsA- overgrown rat gingival tissue. Furthermore, the reduced activity of MMP-1 reported in CsA-treated human gingival fibroblasts was correlated with abrogation of MMP-3 expression, an essential activator of pro- MMP-1, rather than an increase in TIMP-1 levels (Hyland *et al.,* 2003; Bolzani *et al.,* 2000).

On the other hand, Gagliano *et al* (2004) showed that CsA decreased MMP-1 protein expression in human gingival fibroblasts without any significant effect on MMP-1 gene expression. This report also demonstrated that expression of both MMP-2 gene and protein was not affected by CsA. Likewise, Yamaguchi *et al* (2004), Gagliano *et al* (2004) and Dannewitz *et al* (2006) found that levels of TIMP-1 gene expression in human gingival fibroblasts were not significantly modified by CsA treatment.

Additionally, it was revealed that MMP-8 and MMP-9 levels in GCF were not modified by CsA therapy. Instead, their elevation might be related to the gingival inflammation (Atilla *et al.,* 2001).

Effect of CsA on Intracellular Pathway of ECM degradation:

Much attention has been paid to the putative role of CsA in the inhibition of the intracellular collagen degradation. Decreased levels of α2β1 integrin expression have been reported in gingival fibroblasts derived from CsA-hyperplastic gingiva (Kataoka *et al.,* 2003) whilst another study has attributed CsA's inhibitory effects of collagen phagocytosis to perturbation of intracellular calcium signalling, thereby down-regulating the affinity of α2β1 integrin "transmembrane glycoprotein receptors" to type I collagen (Arora *et al.,* 2001).

Additionally, others have shown that the suppressed intracellular degradation of collagen reported in human gingival fibroblasts treated with 200ng/ml CsA has been correlated with decreasing of both cathepsin L expression and activity (Yamada *et al.,* 2000; Yamaguchi *et al.,* 2004). These findings, however, were in line with the clinical observations involving severe gingival hyperplasia reported in both cathepsin L-

deficiency mice (Nishimura *et al.,* 2002) and in the congenital disease- Mucolipidosis II (I-cell disease)- characterized by defect in lysosomal cysteine proteinases function involving cathepsins (Patel & Ambani, 1980; Taylor & Shuff, 1994).

1.5.2. Effect of CsA on gingival cell proliferation:

1.5.2.1 Effect of CsA on gingival fibroblast proliferation:

There is a wealth of conflicting data on the effect of CsA on gingival fibroblast proliferation (Hallmon & Rossmann, 1999; Pisanty *et al.,* 1988; Kataoka *et al.,* 2005). Some *in vitro* studies concur that long-term exposure to CsA may have a stimulatory effect on gingival fibroblast proliferation as reported by Willershausen-Zönnchen *et al* (1992), Marriotti *et al* (1998) and Bartold (1989).

Bartold *et al* (1989) found that 10^{-9}g/ml CsA up-regulated DNA synthesis and the proliferation rate of HGF with most noticeable stimulation being reported in the presence of 10% FBS, and that this capacity was retained even in absence of FBS or in the presence of lipopolysaccharide that usually inhibit proliferation of these cells. In agreement with Bartold's findings, Barber *et al* (1992) demonstrated that addition of CsA to HGFs pre-exposed to a lipopolysaccharide derived from F. nucleatum reversed the inhibitory effect of these bacterial products on DNA synthesis. Chae *et al* (2006) also showed that treatment of serum starved- HGFs with CsA (100 and 500ng/ml) for 6 days significantly activated the cell cycle along with an increase in the cell viability and DNA incorporation of BrdU.

These findings were also in agreement with a flow-cytometric study that showed an increase in the *in vitro* proliferative activity of CsA-treated HGF illustrated by a promoted progression of cell cycle and an increase in the expression level of cyclin B1 (Parkar *et al.,* 2004). Similarly, Kim *et al* (2008) revealed recently that CsA up-regulated *in vitro* both cell viability and BrdU incorporation in HGF cell lines with a maximal increase being found in HGF cells obtained from CsA-induced overgrown gingival tissues. Kantarci *et al* (2007) also demonstrated immunohistochemically that all drug- induced gingival hyperplasia showed an increase in the proliferative activity of gingival fibroblasts. Interestingly, Cotrim *et al* (2003) showed a bimodal effect of CsA on proliferation of human gingival fibroblasts cultured for 24h; low doses of

(CsA $\leq$ 200ng/ml) stimulated human gingival fibroblast proliferation, whilst higher doses of CsA (400-800ng/ml) inhibited proliferation of these cells.

In contrast to the previous findings, Yamaguchi *et al* (2004) found that long-term exposure to a low dose of CsA (200ng/ml) had no effect on viability of human gingival fibroblasts. James *et al* (1995) also revealed that low doses of CsA had no significant effect on proliferation of human gingival fibroblasts. Whilst, extremely high doses of CsA 10^{-5} g/ml induced a significant decrease in human gingival fibroblast proliferation. Furthermore, Jung *et al* (2008) revealed *in vitro* that 24h stimulation with CsA failed to induced an increase in HGF proliferation. However, these discrepancies between studies could be attributed to heterogeneity of cell line strains or to differences in the experimental conditions (incubation period, concentrations of CsA used in each study, FCS concentration).

1.5.2.2. Effect of CsA on gingival keratinocyte proliferation:

Much attention has recently been concentrated on the potential role of gingival keratinocytes in the pathogenesis of CsA-induced gingival hyperplasia but findings are still inconclusive.

Yoshida *et al* (2005) assessed proliferation of cultured rat gingival cells after incubation for 3days and showed that CsA at concentration of 200-800ng/ml induced hyper-proliferation of gingival cells by 23-25% compared to control. These findings were supported by an animal study that showed that treatment with CsA caused buccal epithelial hyperplasia associated with an increase in proliferating cell nuclear antigen (PCNA) expression (Cetinkaya *et al.,* 2006). The same study also reported an increase in epithelial thickness and PCNA expression in both buccal and sulcular epithelium of rats subjected to CsA treatment along with ligature-induced gingival inflammation suggesting that CsA-induced gingival hyperplasia might be mediated by an increase in the proliferative activity of epithelium cells and that dental plaque might aggravate the condition (Cetinkaya *et al.,* 2006).

Similarly, Nurmenniemi *et al* (2001) also demonstrated immunohistochemically that the epithelial hyperplasia reported in CsA-induced gingival hyperplasia was

associated with hyperproliferation in both sulcular and oral gingival epithelium with a maximal proliferative activity being observed in the oral gingival epithelium.

However, Tu *et al* (2006 & 2008) reported two conflicting data; while they found through an animal study that the increase in the epithelial thickness observed in CsA-induced gingival hyperplasia was associated with an increase in cyclin D1 and PCNA expression, their flow cytometric study on human oral epidermoid carcinoma cell lines showed that CsA caused a cell cycle stasis. Furthermore, Birraux *et al* (2006) demonstrated that low concentration of CsA had no effect on the proliferation of an immortalized normal human oral keratinocyte cell line (HOK-16B), primary oral keratinocytes, and epitheloid cervical carcinoma cell line (Hela) cultured for up to 96h. They did report, however, that higher concentration 10^3ng/ml of CsA exhibited an inhibitory effect on proliferation of all cell types. Moreover, inhibition of both primary oral keratinocytes and HOK-16B cell proliferation caused by culture with 10^4ng/ml CsA was maintained for at least two days after withdrawal of CsA. Interestingly, Lauer *et al* (2006) showed both the inhibitory and stimulatory effect of CsA on human gingival keratinocyte proliferation; CsA significantly reduced their proliferation rate from day 3 to day 6 and then stimulated their proliferation from day 6 to day 9 with maximal increase being observed on day 9 at a concentration of 1000ng/ml.

1.5.3. Effect of CsA on gingival cell apoptosis:

Apoptosis, programmed cell death, is stimulated mainly by two characteristic pathways: an extrinsic pathway that is mediated by death receptor (TNF-a & Fas), and an intrinsic or mitochondrial pathway that is regulated by pro-apoptotic proteins such as Bax and Bid and anti-apoptotic proteins such as Bcl-2. Both pathways are executed by a key component referred to as caspase-3 that is activated after being cleaved, leading in the end to cell apoptosis (Fulda & Debatin, 2006).

Apoptosis in conjunction with cell proliferation maintains tissue homeostasis and regulates tissue growth (Shimizu *et al.,* 2002; Alaaddinoglu *et al.*, 2005). However, according to findings reported by Shimizu *et al* (2002) who demonstrated that the increase in epithelial thickness observed in nifedipine-induced gingival hyperplasia mainly results from prolonged survival of cells rather than an increase in

keratinocytes's mitotic activity, Niimi *et al* (1990) also revealed that the epithelial hyperplasia reported in CsA-induced gingival hyperplasia was not correlated with the mitotic activity of keratinocytes but to prolongation of keratinocyte life time through suppression of apoptosis. Moreover, an immunohistochemical study of gingival epithelial specimens obtained from patients with CsA-induced gingival hyperplasia showed a reduction of apoptosis and up regulation of bcl-2 (Bulut *et al.,* 2005). These findings were supported by a recent immunohistochemical study that revealed a decrease level of caspase-3 expression in biopsies of CsA-induced overgrown gingiva indicating that development of CsA-induced gingival hyperplasia might be correlated with inhibition of apoptosis (Bulut *et al.,* 2007).

These observations were consistent with results from studies with HGF. Nishikawa *et al* (1996) suggested that the excessive number of fibroblasts in drug-induced gingival hyperplasia may be caused by inhibition of apoptosis. Similarly, Kantarci *et al* (2007) found through an immunohistochemical study of connective tissue biopsies obtained from CsA/phenytoin/ and nifedipine-induced overgrown human gingiva that gingival fibroblasts showed a decrease level of caspase-3 expression compared to control indicating that the decrease in apoptosis rate might play an important role in accumulation of gingival fibroblast being observed in all forms of drug-induced gingival hyperplasia.

Furthermore, these findings were supported by a recent *in vitro* report that revealed that stimulation of HGF with CsA for 24h caused a significant decrease in apoptosis rate through both the intrinsic and extrinsic pathways suggesting that the decreased level of apoptosis might play a more significant effect than the increase in cell proliferation in increasing the HGF number being reported in this study (Jung *et al.,* 2008).

In contrast, a recent *in vitro* study revealed that CsA increased the apoptosis of human gingival squamous carcinoma cell line through the mitochondrial pathway by increasing the expression level of Bax and caspase 3 protein (Tu *et al.,* 2009) However, Alaaddinoglu *et al* (2005) reported no differences in the levels of keratinocyte apoptosis in patients with CsA-induced gingival hyperplasia and systemically healthy individuals but experiencing gingivitis. Birraux *et al* (2006) also

found that survival of both CsA-treated oral keratinocyte cell lines and primary oral keratinocytes was not associated with changes in apoptosis levels either at intracellular bcl-2 levels or TNF-α- induced apoptosis.

1.5.4. Effect of CsA on cytokine release:

Cytokines and chemokines such as IL-1α, IL-1β, IL-6 and IL-8 play a significant role in periodontal diseases (Okada & Murakami, 1998) and growing attention has been paid to their potential role in the pathogenesis of CsA-induced gingival hyperplasia with conflicting results.

Myrillas *et al* (1999) reported that incubation of normal and overgrown gingival fibroblasts for 24h with different concentrations of CsA released no detectable amount of IL-1β compared to control, whereas production of IL-6 in all fibroblast strains was suppressed in a dose-dependent manner with the maximal inhibition being reported at 2000ng/ml CsA. In contrast, Chae *et al* (2006) demonstrated that 500ng/ml CsA up-regulated production of IL-6 and transforming growth factor (TGF-β1) in human gingival fibroblasts in a time-dependent manner with the maximal release being reported at 24h. But, interestingly, it has been shown that neutralisation of TGF-β1 resulted in a significant reduction in expression of CsA-induced IL-6 while release of CsA-induced TGF-β1 was not affected by blocking IL-6. These findings suggest that TGF-β1 is an essential regulator of CsA-induced IL-6 release in human gingival fibroblasts. Additionally, it has been reported that human gingival fibroblasts treated with IL-6 or TGF-β1 exhibited an increased rate of proliferation compared to control. Furthermore, neutralizing IL-6 and TGF-β1 caused downregulation of CsA-induced HGF proliferation rate (Chae *et al.,* 2006; Cotrim *et al.,* 2003).

Leonardi *et al* (2001) found that the exposure to CsA for 24h caused a significant increase in IL-6 and IL-8 release with a parallel decrease in IL-1β release along with a reduction in cultured human conjunctival fibroblasts viability; they related their findings to the toxic activity of the high doses used of CsA.

Hyland *et al* (2004) reported that release of both keratinocyte growth factor KGF and scatter factor SF was increased in both normal and overgrowth gingival fibroblast strains treated with 2000ng/ml CsA. Likewise, Das *et al* (2002) revealed that normal

gingival epithelial cells incubated with 500ng/ml CsA for 3days expressed higher levels of KGFR –keratinocyte growth factor receptor- compared to control. The authors concluded that the increased level in KGFR is consistent with the increase in proliferation rate of epithelial cells observed in CsA-induced gingival hyperplasia.

Chin *et al* (2006) demonstrated that CsA caused up-regulation of both EGF "epidermal growth factor" and EGFR "epidermal growth factor receptor " in human oral epidermal carcinoma cell lines with the greatest stimulation being reported at concentration 100ng/ml .

The effect of CsA on IL-1α and IL-8 has received little direct attention, although it has been suggested that pathogenesis of CsA-induced gingival hyperplasia may involve alteration in the expression levels of various inflammatory cytokines in the gingiva (Ruhl *et al.,* 2004) who found an increase in the salivary levels of IL-1α, IL-6, and IL-8 in patients with CsA-induced gingival hyperplasia. IL-1α, however, may be a co-factor for nifedipine-induced gingival hyperplasia (Sato *et al.,* 2005) and increased IL-8 levels were reported in cultured HGFs treated with phenytoin (Modéer *et al.,* 2000).

1.6 Summary and aims of study:

The mechanism of CsA-induced gingival hyperplasia is still unclear despite numerous studies being conducted in an attempt to understand the pathogenesis of the condition. Most studies have focused on the role of connective tissue- CsA interaction with conflicting results either in terms of the effect of CsA on gingival fibroblast proliferation or on ECM metabolism. More recently, however, attention has focused on the potential role of gingival keratinocytes along with other locally released mediators as well as the imbalance between cell proliferation and apoptosis in CsA-associated gingival hyperplasia. The potential role of dental plaque in the development of the condition has also been examined, again with inconclusive results. This may reflect the complexity of the *in vivo* conditions, and it is still unclear whether the accumulation of dental plaque is a consequence of gingival changes caused by the drug itself or an essential factor for initiating the condition.

Despite the lack of clear evidence it is generally agreed that the pathogenesis of CsA-induced gingival hyperplasia is multifactorial including complex interactions between the gingival tissue, drug, genetic factors, dental plaque in addition to other locally released mediators such as growth factors and cytokines.

There have been very limited studies *in vitro* investigating whether the pre-exposure to bacterial products might modulate the response of human gingival fibroblasts and keratinocytes to CsA. Therefore, the aims of the present study were to investigate *in vitro*:

1- Whether CsA at clinically-relevant concentrations might have direct effects on cell viability and release of proinflammatory cytokines including IL-1α, IL-6, and IL-8 in monolayer cultures of human gingival fibroblasts and keratinocytes;

2- Whether the pre-exposure to bacterial products might modulate the response of monolayer cultures of the studied cells to CsA in terms of cell viability and the cytokine release;

3- The effect of CsA alone or in combination with bacterial products on proliferation and apoptosis of monolayer cultures by a novel multi-parameter flow-cytometric analysis;

4- The response of multilayer cultures of gingival and buccal epithelium to CsA and bacterial products in terms of tissue viability, release of the inflammatory cytokines, proliferative activity and apoptosis.

Chapter Two

Materials and Methods (Monolayer cultures)

This chapter describes the main experimental methods used to study the effect of CsA with the monolayer cultures. Any variations in the techniques used will be discussed in the appropriate chapters.

2.1 Cells used in study:

Four cell lines were used in the study:

human gingival keratinocytes (FIBS),

human buccal carcinoma keratinocytes (TR146),

immortalized human oral keratinocytes (OKF6)

human gingival fibroblasts (HGF),

FIBS, TR146 and OKF6 lines were obtained from Centre for Clinical and Diagnostic Oral Sciences (CDOS), Barts and The London School of Medicine and Dentistry, London. Upon receipt, the cells were thawed, plated in T-75 Flasks (Nunc.dk) and cultured in medium supplemented with 10% FBS and 1% penicillin-streptomycin (see Table 2.1).

HGF cells were purchased from American Type Culture Collection (ATCC) (HGF-1, reference code: CRL-2014). All cell lines used in this study were between passage 10 and 20.

Cells were then incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were routinely washed with sterile phosphate buffered saline (PBS) and fed with fresh medium twice a week. When the cells reached 80% confluence, they were harvested with 0.2% trypsin and EDTA (Gibco Invitrogen. UK) for freezing or secondary cultures.

2.2 Cyclosporin (CsA) preparation:

CsA (25mg) (Sigma-Aldrich, Poole, UK) was dissolved in 25ml absolute ethanol (Sigma UK) to make a 1mg/ml stock solution and stored in 1ml aliquots in -80^{o}C until use. The experimental CsA concentrations were then prepared by individually diluting 20µl, 10µl, 5µl and 1µl of this stock in 10ml of DMEM yielding a final CsA concentration of 2000ng/ml, 1000ng/ml, 500ng/ml, and 100ng/ml respectively.

Cell Line	Origin	Source	Culture Medium
Keratinocyte			
TR146	human buccal carcinoma keratinocytes	Centre for Clinical and Diagnostic Oral Sciences, Queen Mary University, London	DMEM with 4.5g/L glucose + L-glutamine
FIBS	Human gingival keratinocytes	Centre for Clinical and Diagnostic Oral Sciences, Queen Mary University, London	As above
OKF6	Immortalized human oral keratinocytes	Centre for Clinical and Diagnostic Oral Sciences, Queen Mary University, London	As above
Fibroblast			
HGF	Human gingival fibroblasts	American Type Culture Collection (ATCC)	DMEM +4.5g/L glucose +L-glutamine +Pyruvate

Table 2.1 Cell lines used in the study

2.3. Treatment:

2.3.1 Short-term:

Cells were seeded into 24- well plates (Falcon) at a density of 4 x 10^4cell/well and allowed to settle down for 24hour. Then, cells were washed once with phosphate buffered saline (PBS) and starved for 24h by replacement of the medium by serum-Free DMEM.

Following serum starvation, cells were again rinsed with PBS and stimulated with increasing concentrations of CsA: 100ng/ml, 500ng/ml, 1000ng/ml, 2000ng/ml and control (medium only) in FBS-free DMEM for various time intervals; 4h, 6h, 8h, 12h, and 24h. Supernatants were then collected and stored at -80^{o}C until use for measuring cytokine contents by Enzyme-linked immunosorbent assay (ELISA). After washing with PBS, cell viability was evaluated at the end of each time point by MTT assay. All experiments were carried out three times in triplicate wells.

2.3.2 Long-term:

Cells were seeded into 24-well plates at a density of 4 x 10^4cell/well in DMEM containing 10% FBS and allowed to attach to the well for 24 hours. Cells were then

starved by incubation in FBS-free DMEM for further 24 hours. They were then stimulated for 6 days with FBS-free DMEM containing various concentrations of CsA; 100ng/ml, 500ng/ml, 1000ng/ml, 2000ng/ml and 0 (only medium).The culture media was collected every other day with re-adding CsA at the same concentrations. In other words, supernatants were collected on day 2, 4, and 6 and frozen at – 20^{o}C until use for measuring cytokine contents. Cells were washed with phosphate-buffered saline (PBS) with every medium changing. Cell viability at the end of the experiment was assessed using MTT (see section 2.4.1) assay. All experiments were carried out three times in triplicate wells.

2.4. Cell viability assays:

The MTT assay (Mosman, 1982) has been applied to determine the effect of CsA on viability of cultured human gingival keratinocyte, human oral keratinocyte, and human gingival fibroblast cell lines. This assay depends on measuring the metabolic activity of viable cell's mitochondrial dehydrogenases presented in their ability to reduce the (tetrazolium salt 3[4,5-3 dimethylthiazol-2-YL]-2,5-diphenyltetrazolium Bromide) (MTT) to an insoluble blue formazan dye. This analysis was carried out as follows:

1. 500µl of MTT reagent (final concentration: 0.5mg/ml in PBS) was added to each well and plates were then incubated for 1h in a humidified atmosphere at 37^{o}C in the dark
2. Plates were aspirated and rinsed with PBS.
3. 500µl of acidified isopropanol (25µl of 0.04N HCL in 20ml isopropanol) was then added to each well. The plates were incubated for 1h to elute any formazan crystals that had formed.
4. The optical density (OD), which is known to be directly proportional to the number of living cells (Yoshida *et al.,* 2005), was measured at 570nm using a micro plate reader (Optima). Cell viability was expressed as the absorbance at 570nm: *Cell viability = OD (570nm) test / OD (570nm) control x 100*

2.5 Enzyme-linked immunosorbent assay (ELISA):

IL-1α, IL-6, IL-8 release was determined by sandwich Elisa (Duo Set®; R & D systems, Abingdon, UK). Supernatants were defrosted for testing and the level of cytokines were measured according to the manufacturer protocol as outlined below:

1. 96-well Elisa plates were coated with 100µl per well of the respective capture antibody (mouse anti-human cytokine) diluted in PBS without carrier protein. The plates were then sealed and incubated overnight at room temperature.
2. Plates were then aspirated and washed by filling each well with 400µl washing buffer (0.05% Tween®20 in PBS, PH 7.2-7.4) to remove unbound antigen. Complete removal of the content was attained by inverting and tapping the plates on to a clean, absorbent paper. This process was repeated twice for a total of three times.
3. Thereafter, plates were blocked with 300µl per well of either reagent diluent (1% BSA in PBS, PH 7.2 - 7.4) for IL-1α and IL-6 or with block buffer (1% BSA in PBS with 0.05% NaN_3) for IL-8 and then incubated at room temperature for 1h.
4. The plates were again aspirated and washed three times with washing buffer.
5. 100µl of standards in reagent diluent (prepared according to manufacturer instructions) or samples were then added to the appropriate plate wells which were sealed with an adhesive strip and incubated for further 2h at room temperature. Standards were run in duplicate while the samples were run in triplicate.
6. Aspiration and washing then were performed as described above.
7. 100µl / well of detection antibody, a specific biotin- conjugated goat anti human cytokine (IL-1α or IL-6 or IL-8) diluted in reagent diluent was added to each well. Plates were sealed with a new adhesive strip and incubated for additional 2h at room temperature.
8. In order to remove unbound cytokines and other components of the samples, Plates were thoroughly aspirated and washed three times with washing buffer as described above.
9. Subsequently, the quantitative determination of the amount of tested cytokines in the samples were performed by adding 100µl of the streptavidin conjugated to horseradish- peroxidase (HRP) diluted in reagent diluent to each well, plates

were then sealed and incubated for 20 minutes at room temperature in the dark.

10. Aspiration and washing were performed as described above to remove all unbound Avidin-HRP conjugate.
11. A colour reaction was stimulated by adding 100µl of TMB (tetramethylbenzidine) substrate solution (Sigma.UK) to each well. This reaction which exhibits as a change in colour and develops only in those wells that contain the specific cytokine was allowed to develop over a short incubation period (20 minutes) at room temperature in the dark.
12. The colour reaction was terminated by adding 50µl 2N H_2SO_4 to each well. The plates were gently tapped to ensure through mixing.
13. Finally, the optical densities OD were measured immediately at wave length of 450nm by using a micro plate reader.

2.5.2 Calculation of cytokine release:

A standard curve for each set of measured samples was created using Microsoft Excel by plotting the log of cytokine concentrations on the X-axis against the log of corresponding OD (absorbance) for each standard plotted on the Y-axis (Fig 2.1). However, by completing a regression analysis of the points, a straight line of best fit can then be determined. This line shows a direct relationship between cytokine concentrations and the corresponding absorbance and can be represented by the equation:

$$Y = Mx + C$$

Where: M; the gradient of the line and C: a constant

Practically, M and C can be determined from the standard curve. The ODs of the evaluated samples were already measured. Hence, the concentration (X) of cytokine produced in pg/ml in the tested samples can be calculated by adjusting the previous equation as follows: $X = (Y-C)/M$.

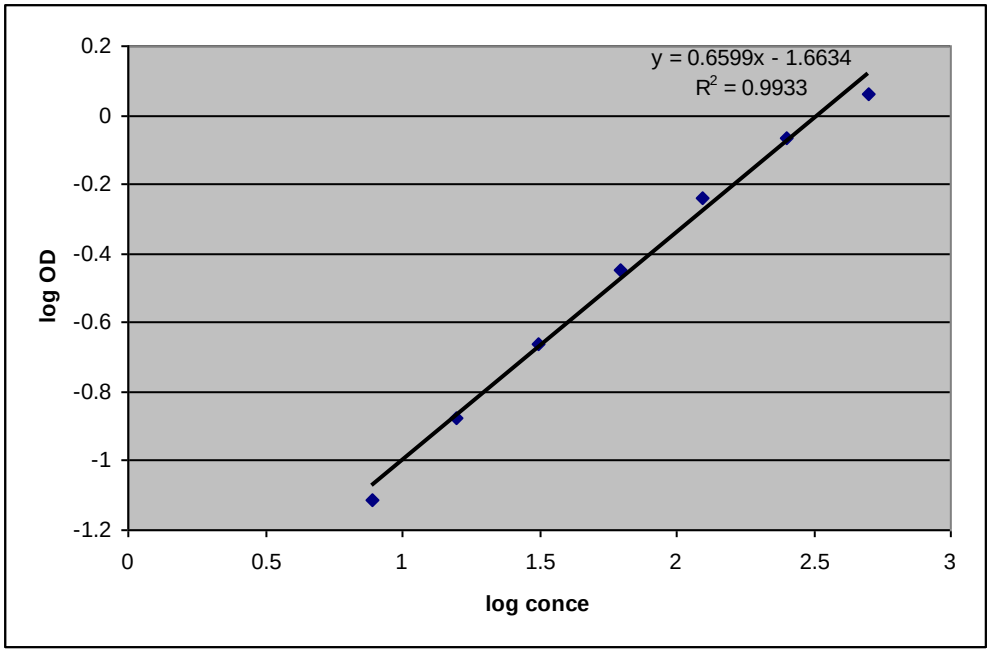

Figure 2.1. IL-1α Standard curve for short-term treated OKT6 cell line.

2.6. Bacterial strains and culture conditions:

The bacterial strains used in this study are shown in Table 2.2. *P. gingivalis* W50 strain and *F. nucleatum* ATCC12104 strain were maintained on blood agar (BA) plates made from sterile blood agar base No.2 (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood in an anaerobic cabinet (Don Whitely scientific, West Yorkshire, UK) containing 80% N_2, 10% H_2 and 10% CO_2 at 37^{o}C for one week. Colonies (3-4days old) were subcultured in 10 ml of brain heart infusion broth (Oxoid Ltd, Basingstoke, UK) supplemented with haemin (5µg ml^{-1}) and incubated for 24hrs.

P. intermedia T558 strain was cultured on fastidious anaerobic agar (FAA) (Lab M, Bury UK) supplemented with 6% defibrinated horse blood (TCS Bioscience Ltd, Buckingham, UK) and 0.05µg/ml sterile haemin (from a 5 µg/ml stock solution) in an anaerobic atmosphere of 80% N_2, 10% H_2 and 10% CO_2 at 37^{o}C. Colonies (3-4days old) were subcultured in 20 ml of brain heart infusion broth (Oxoid) supplemented with haemin (0.05µg/ml) and incubated for 48hrs.

A. actinomycetemcomitans 280 strain was also cultured on fastidious anaerobic agar (FAA) (Lab M, Bury UK) supplemented with 6% defibrinated horse blood (TCS

Bioscience Ltd, Buckingham, UK) and 0.05µg/ml sterile haemin (from a 5 µg/ml stock solution) in an aerobic atmosphere of 6% CO2 at 37^{o}C. Colonies (3-4days old) were sub cultured in 20 ml of brain heart infusion broth (Oxoid Ltd, Basingstoke, UK) supplemented with 1.0 mg/ml NaHCO3 and incubated for 48hrs.

The purity of plate cultures was confirmed by performing Gram stains.

Cells were pelleted by centrifugation at 10,000 rpm for 15 minutes at 4^{o}C and cell-free supernatants were collected, filter sterilized using 0.22µm disposable Millex-GV PVDV low protein binding filters (Millipore Corporation, Bedford MA, USA) and stored at -80^{o}C until use.

Species	Supplied by
Fusobacterium nucleatum ATCC12104	Dr. A.Hashim Centre for Infectious Diseases
Porphyromonas gingivalis W50	Dr. A.Hashim
Prevotella intermedia T588	Dr. R.Whiley CDOS
Aggregatibacter actinomycetemcomitans 280	Dr. R.Whiley

Table 2.2. Bacterial strains used in this study

2.7 Treatment with bacterial supernatants:

Cell lines (TR146, OKF6, FIBS and HGF) were seeded into 96 well plates at density of (5 x 10^3 cell/well) in 100µl DMEM containing 10% FBS. Cells were then allowed to settle down and attach to the well by incubation at 37^{o}C and 5% CO_2 for 24h. Cells were washed twice with PBS and then starved for 24h by incubation in serum-free DMEM at 37^{o}C and 5% CO_2.

After one wash with PBS, cells were stimulated by different concentrations (0, 1/10, 1/50 and 1/100) of bacterial supernatants (*P.g*, *A.a*, *F.n*, and *P.i*) in 100 µl serum-free DMEM for 24hours.

The potential toxicity of the bacterial supernatants on the cell lines was assessed using an Alamar Blue assay and a LDH assay.

2.8 Alamar Blue assay:

Cell lines (TR146, OKF6, FIBS and HGF) were seeded into 96 well plates at density of (5*1000 cell/well) in 100µl DMEM containing 10% FBS. Cells were then allowed to settle down and attach to the well by incubation at 37^{o}C and 5% CO_2 for 24h. Cells were washed twice with PBS and then starved for 24h by incubation in serum-free DMEM at 37^{o}C and 5% CO_2.

The cells were stimulated by different concentrations (0, 1/10, 1/50 and 1/100) of bacterial supernatants (*P.g, A.a, F.n, and P.i*) in 100 µl serum-free DMEM containing 10% Alamar Blue solution (Invitrogen.uk). Cultures were then incubated for further 24h at 37^{o}C and 5% CO_2 where fluorescence intensity was then measured by Elisa reader using excitation 480nm. Cell viability was calculated as follows:

Cell viability = fluorescence test/ fluorescence control x 100

2.9 LDH assay:

The effect of the bacterial supernatants on LDH release by the cells was measured using an *in vitro* Cytotoxicity Detection Kit^{plus} (LDH) (Roche, Mannheim, Germany).

TR146, OKF6, FIBS and HGF were seeded into 96 well plates at density of (5*1000 cell/well) in 100µl of Pyruvate-free DMEM containing 10% FBS. Cells were then allowed to settle down and attach to the well by incubation at 37^{o}C and for 24h. Cells were washed twice with PBS and then starved for 24h by incubation in serum-free DMEM at 37^{o}C and 5% CO_2.

Cells were then washed with PBS and simulated for 24hour by different concentrations (0, 1/10, 1/50 and 1/100) of bacterial supernatants (P.g, A.a, F.n, and P.i) in Pyruvate and serum-free DMEM in addition to controls (background control: cell-free medium, low control: medium containing un-treated cells, high control: totally lysed cells). Afterwards, cells in high control were totally lysed by adding 5 µl lysis buffer for 5 minutes and then 100 µl reaction mixture (250µl of catalyst mixed

with 11.25ml of dye solution) was added to all cell cultures including controls and incubated in the dark at room temperature for 30 minutes. The detection reaction was terminated by adding 50µl stop solution and absorbance was immediately measured by Elisa reader filter 490nm. Cytotoxicity was expressed as changes in the optical density compared to that of high control as follows:

% Cytotoxicity = OD (test) - OD (background)/OD (high control)-OD (background) x 100

2.10. Multicolour flow cytometry:

2.10.1 Reagents and antibodies:

Reagents and antibodies used in this study are shown in Table 2.3.

Reagents and antibodies	**Source**
Edu click-it pacific blue flow cytometry kit	Invitrogen Ltd, Paisley, UK
fix & perm Cell permeabilization kit	Invitrogen Ltd, Paisley, UK
Alexa fluor 647-conjugated mouse anti-IgG1 isotype control	Invitrogen Ltd, Paisley, UK
Alexa fluor 488-conjugated mouse anti-IgG2b isotype control	Invitrogen Ltd, Paisley, UK
Alexa fluor 488-conjugated mouse anti-IgG1 isotype control	Invitrogen Ltd, Paisley, UK
Alexa fluor 488-conjugated mouse anti-human Bax (clone 2D2)	Santa Cruz Biotechnology, CA, USA
Alexa Fluor 647-conjugated mouse anti-human Bcl-2 (clone C-2)	Santa Cruz Biotechnology, CA, USA
Alexa Fluor 488-conjugated mouse anti-human Cyclin D1 (clone A-12)	Santa Cruz Biotechnology, CA, USA
Alexa Fluor 647-conjugated mouse anti-human Cyclin B1 (clone D-11)	Santa Cruz Biotechnology, CA, USA

Table 2.3. Reagents and antibodies used for the FACS study

2.10.2. Cell treatment:

OKF6, FIBS, TR146 and HGF grown in 75cm flask up to 90% confluence were trypsinized and seeded at density of 5 x 10^5 into T75 flasks. HGF cells were seeded at density of 2.5 x 10^5 into T25 flasks. Cell lines were cultured in DMEM containing 10% FBS for a further 24h.

After washing twice with PBS, cells were serum starved for 24h by incubating in serum-free DMEM to synchronise cells in the G0 phase of cell cycle (Takeuchi. 2004). Cells were washed again and then either re-incubated in serum-free DMEM or stimulated for 24h with 1/100 bacterial supernatants in serum-free DMEM (Table 4.2). Afterwards, cells were washed as previously. At this time point, serum starved untreated cells were either re-incubated with serum-free DMEM (control for both Bax, Bcl-2 and cyclin expression), or stimulated with DMEM containing10% FBS (as a positive control for cell cycle and DNA synthesis), or challenged with either 2000ng/ml or 250ng/ml CsA. However, bacterial supernatant-pre stimulated cells were then either re-challenged with bacterial supernatants only or with a combination of bacterial supernatant plus 2000ng/ml or 250ng/ml CsA for further 24hr where the experiment was terminated and cells were then prepared for flow cytometry analysis.

2.10.3. Controls:

Three types of control were included in each experiment:

1. *Biologic controls*: including stained-untreated cells. These controls allow determining the biological effect of CsA and bacterial supernatants comparing with these controls.
2. *Isotype matched negative controls*: these controls have the same immunoglobulin type and conjugated with same fluorochrome as the primary antibodies under investigation. They are used to determine the background (autofluorescence, un-specific binding).
3. *Compensation controls:* also known as fluorochromes minus one controls including the un-treated cells stained with all fluorochromes under investigation minus one. These controls are used to determine the spectral overlap of fluorochrome.

2.10.4. Antibody panels design:

Eight panels of antibodies were set up as shown in Table 2.4. Each panel contained 1µg concentration of an appropriate antibody diluted in the permeabilization medium with a total volume of 100 µl.

Panel	Cyclin B1 Antibody 1µg	Cyclin D1 Antibody 1µg	Bax Antibody 1µg	Bcl-2 Antibody 1µg	Alexa flour 647 isotype Antibody 1µg	Alexa flour 488 isotype Antibody 1µg	Alexa flour 488 isotype Antibody 1µg
1	√	√					
2	√						
3		√					
4					√	√	
5			√	√			
6			√				
7				√			
8					√		√

Table 2.4. Antibody panels used in for the FACS study

2.10.5. Protocol for Cell cycle , Edu and cyclin staining:

Labelling cells with EdU:

1. After washing twice with PBS, Cells were incubated for 1hr with 10µM Edu suspended in the culture medium at 37^{o}C, 5% CO_2. EdU-un labelled cells from the same population have been included as a negative staining control.
2. The supernatant was then aspirated and cells were washed with FACS buffer (PBS containing 5% FBS and 0.1% sodium azide).
3. Cells were harvested with trypsin and suspension was centrifuged at 1500rpm for 5 minutes at 4^{o}C.

Cell fixation:

1. Supernatant was removed and cells were resuspended at density 1 x 10^6 cells/ml in FACS buffer.
2. 100µl aliquot of each cell suspension was transferred to polystyrene round-bottom 12 x 75cm Falcon tubes.
3. 100µl Fixation Medium (Reagent A) (fix & perm Cell permeabilization kit, Invitrogen, UK) was added to the flow tube. Cells were incubated for 15 minutes at room temperature in the dark.
4. Cells were washed with 3ml FACS buffer, centrifuged at 1500 rpm at 4^{o}C for 5 minutes. Supernatant was then discarded and pellet was resuspended in 50µl FACS buffer.

Cell permeabilization and staining for flow cytometry:

1. 100µl of the permeabilization medium (Reagent B) containing the appropriate fluorochrome- conjugated antibody cocktail was then added to the matched tube, mixed gently by vortexing and incubated for 30 minutes at room temperature in the dark.
2. Each sample was then washed with 3ml of the FACS buffer and centrifuged at 1500 rpm at 4^{o}C for 5 minutes and the supernatant was discarded.
3. Pellet cells were then dislodged and incubated with Click-it reaction cocktail (0.5ml/each tube) for 30 minutes at room temperature in the dark.
4. Samples were washed once with 3ml FACS buffer and centrifuged for 5 minutes at 4^{o}C at 1500 rpm. Supernatant was discarded.
5. Cells were resuspended in 0.5ml FACS buffer.
6. 5µl Ribonuclease A (component L) was added to each tube and then mixed gently.
7. 2µl of the cell cycle 488-red dye 7-aminoactinomycin D (7-AAD) was added to each tube and mixed well.
8. Samples were incubated for 15 minutes at room temperature.
9. Cell cycle, Edu-labelled cells (Phase S) and expression of cyclin D and cyclin B were then analyzed by flow cytometry.

2.10.6. Protocol for Bax and Bcl-2 staining:

1. Cells were harvested with trypsin and suspension was centrifuged at 1500rpm for 5 minutes at 4^{o}C.
2. Cells were fixed and washed as described above.
3. 100µl of the permeabilization medium (Reagent B) containing the appropriate fluorochrome- conjugated antibody cocktail was then added to the matched tube, mixed gently by vortexing and incubated for 30 minutes at room temperature in the dark.
4. Each sample was then washed with 3ml of the FACS buffer and centrifuged at 1500 rpm at 4^{o}C for 5 minutes and the supernatant was discarded.
5. Samples were resuspended in 500µl FACS buffer and expression of Bax and Bcl-2 was then measured immediately by flow cytometry machine.

2.10.7. Flow cytometric analysis:

Flow cytometry is a technology of quantitative single particles analysis that allows simultaneous analysis of multiple biologic markers of single cells. It is based on the essential principle that single cells labelled with fluorescent dyes and suspended in a fluid stream can be individually analysed as they pass through a light laser beam directed onto a very small zone known as the "interrogated region". At this point, each suspended cell scatters the light in different ways (forward scatter -FSC- and side scatter -SSC) and the fluorescent dye bound to the cells is excited and emits light at a higher wavelength than the light source. The light signals are collected by detectors and analysed to provide information about the chemical and physical structure of each single cell. FSC depends on the cell size and SSC correlates with cell granularity (Brown & Wittwer, 2000).

In this study, a BD FACS Canto II equipped with three lasers, an argon ion Laser emitting 20mW at 488-nm, Red HeNe (15mW, 633-nm) and a violet laser (59mW, 405-nm) (BD Bioscience) was used. Data analysis was performed using the BD FACS Diva software version 6.1.2 (BD Bioscience).

Six parameters (FSC, SSC, PerCp-Cy5.5, Pacific blue, FITC, and APC) were analyzed from a cell sample stained with the Edu pacific blue, 7-AAD, Alexa fluor 488- conjugated cyclin D antibody and Alexa fluor 647-conjugated cyclin B antibody, while four parameters (FSC, SSC, FITC, and APC) were analysed from a cell sample stained with Alexa fluor 488-conjugated Bax antibody and Alexa fluor 647-conjugated Bcl-2 antibody.

Forward scatter (FSC) and side scatter (SSC) were collected using a 488nm band pass filter. FITC (Alexa flour 488) green fluorescence was excited with the 488nm blue argon laser and collected in the 530/30nm channel, APC (Alexa flour 647) red fluorescence was excited with the 633-nm red Diode laser and collected in the 660/20nm channel, Pacific Blue was excited with the 405nm violet laser and collected in the 450/50 channel, PerCep-Cy5.5 (7-AAD) red fluorescence was excited with the 488 nm blue argon laser and collected in the 670LP channel.

For analysis, isotype matched negative controls for all of the antibodies studied were run first to measure the non-specific binding (autofluorescence) and subsequently to determine the position of the positive and negative populations. Compensation for spectral overlap was set using single fluorochrome-labelled samples plus fluorochromes-minus one controls. The forward scatter (FSC) –versus- side scatter (SSC) dot plot was used to exclude cell debris and doublets. 10.000 to 30.000 cells/ sample within each gate were collected using the "medium" setting for sample flow rate. The gated population were then plotted to investigate the fluorescence signals of interest and shown as one parameter histogram displaying the intensity of the fluorescent signal reported on the X-axis against the number of events on the Y-axis or/and two-parameter displays (dot plots) showing two parameters with either linear or logarithmic axes that extend over a four to five decade ranges representing cells with fluorescence values. The vertical or/and the horizontal markers were set on each histogram using the isotype matched negative controls for the antibodies of interest. Data were reported as a percentage of positive cells from both the total population and the singlet gated one.

2.11. Statistical Analysis:

Data were analysed by using One-way ANOVA test along with Student's independent t-test. Statistical significance was considered when $p<0.05$. Findings were further confirmed using SPSS program by one-way ANOVA and Post hoc test.

Chapter Three

Effect of CsA on cell viability and cytokine expression in monolayer cell cultures

3.1. Introduction:

This Chapter describes the effect of different doses of CsA on cell viability and cytokine expression in human gingival keratinocyte cell line, human oral keratinocyte cell line, and human gingival fibroblast cell line at different time intervals in an attempt to determine both the optimal doses of CsA and time intervals of challenging to be used in subsequent *in vitro* experiments.

The cell lines were treated with 0,100, 500, 1000 and 2000ng/ml CsA for up to 144 h (6 days). Cell viability was assessed by MTT assay after 4, 6, 8, 24 and 144h. Cytokine release was measured by ELISA at 4, 6, 8, 24, 48, 96 and 144h.

3.2. Effect of CsA on cell viability:

3.2.1 FIBS cells:

Stimulation of FIBS with CsA caused two distinctive dose-dependent effects: 100ng/ml CsA caused a slight but significant increase in cell viability at 12 and 24h (Fig 3.1 $p < 0.05$ compared to control). Conversely, CsA at doses ≥1000ng/ml caused a slight but significant reduction in the cell viability starting from 6h onwards with a maximal decrease being observed with 2000ng/ml CsA. 500ng/ml CsA had no significant effect on the cell viability.

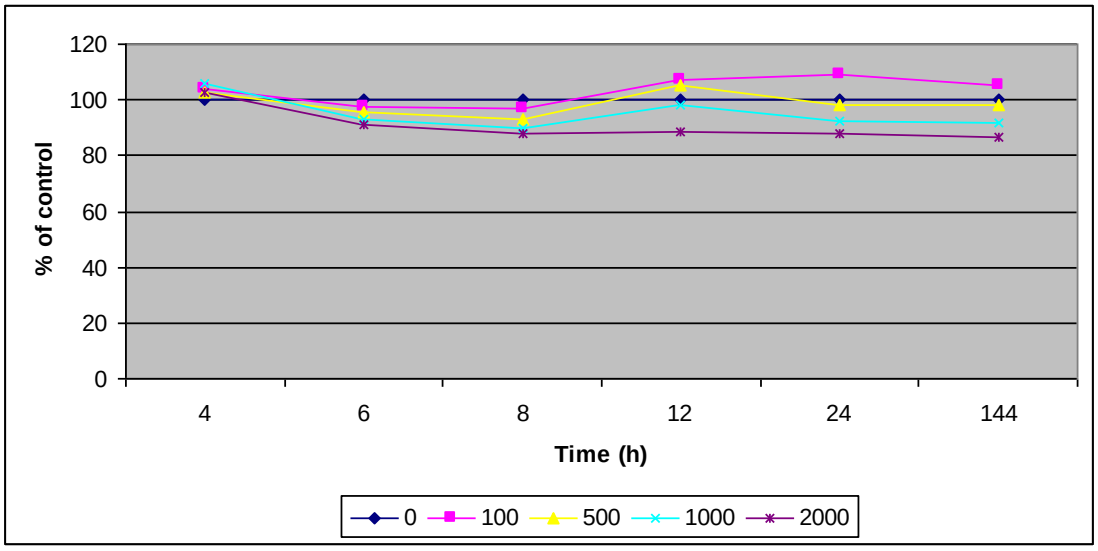

Figure 3.1. Effect of CsA on viability of FIBS.
FIBS were stimulated with increasing doses of CsA and cell viability was measured by MTT assay. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.2.2 OKF6 cells:

CsA had no effect on cell viability in the early hours of stimulation compared to control (Fig 3.2). However, after 8h of stimulation, CsA caused a slight but dose-dependent increase in the cell viability with a maximal increase being observed at 24h with 2000ng/ml CsA ($p < 0.05$). Prolonged exposure to high doses (1000 and 2000ng/ml) caused a significant reduction in cell viability ($p < 0.05$).

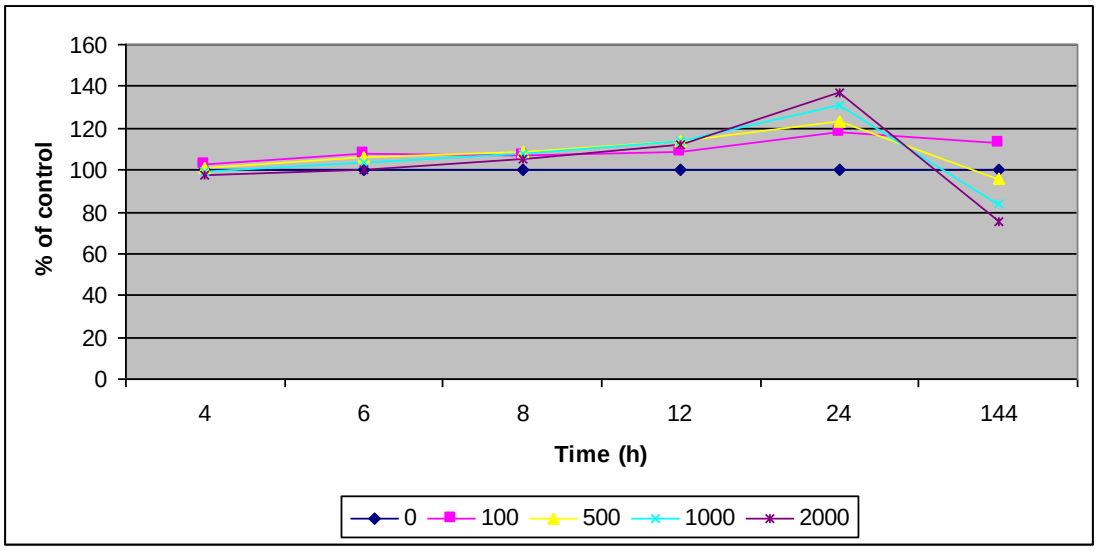

Figure 3.2. Effect of CsA on viability of OKF6.
OKF6 were stimulated with increasing doses of CsA and cell viability was measured by MTT assay. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.2.3 TR146 cells:

CsA had no effect on cell viability up to 24h (Fig 3.3). Prolonged exposure (144h) to higher doses (1000 and 2000ng/ml) however, caused a slight but significant reduction in viability ($p < 0.05$) compared to control.

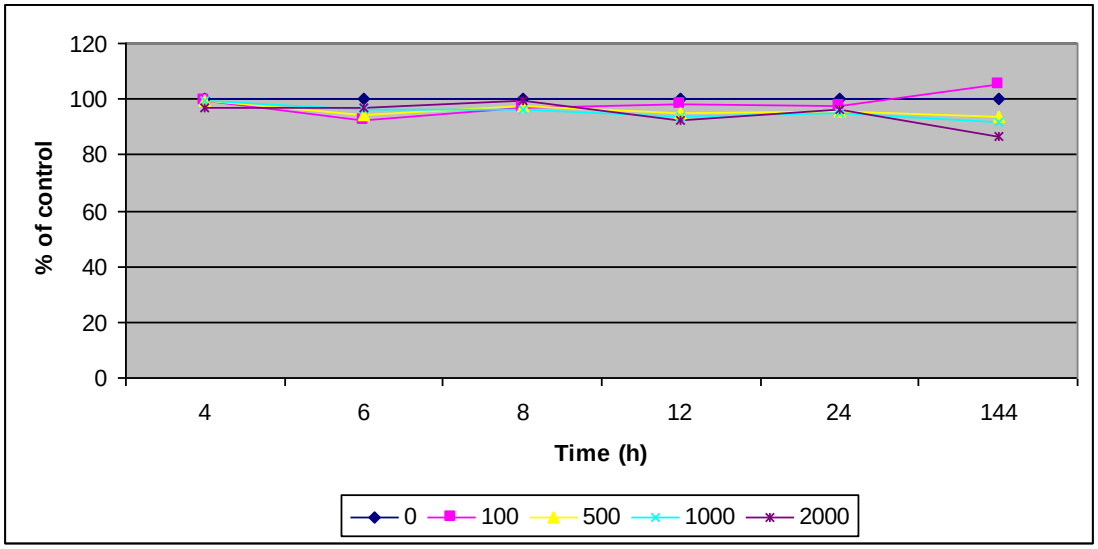

Figure 3.3. Effect of CsA on viability of TR146.
TR146 were stimulated with increasing doses of CsA and cell viability was measured by MTT assay. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM).); n = 9. SEM has been omitted for clarity.

3.2.4 HGF cells:

CsA at doses ≤1000ng/ml caused a slight but significant increase in cell viability at 24h ($p < 0.05$ versus control) but had no significant effect at the other intervals studied (Fig 3.4).

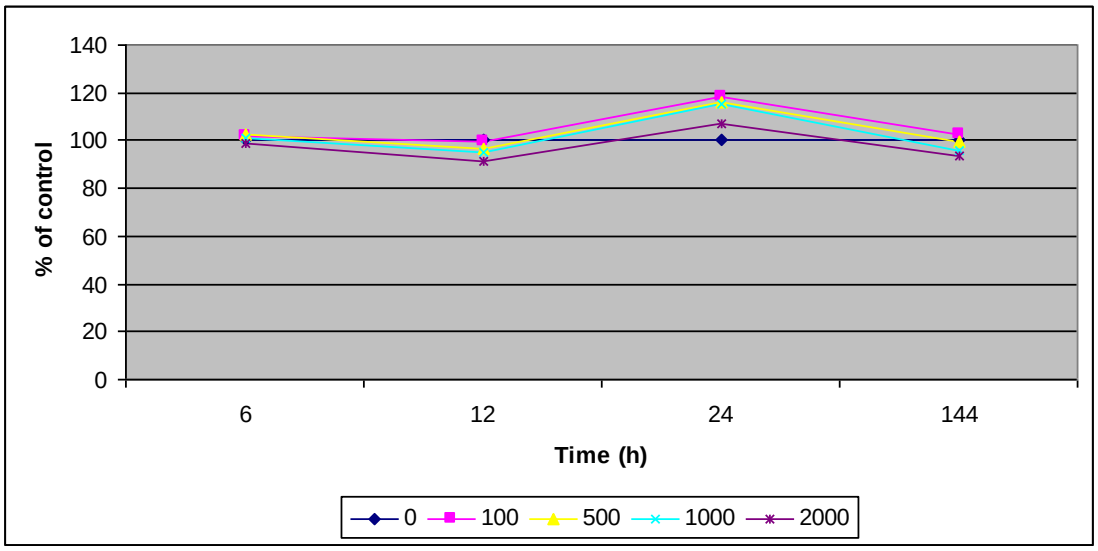

Figure. 3.4. Effect of CsA on viability of HGF.
HGF were stimulated with increasing doses of CsA and cell viability was measured by MTT assay. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.3. Effect of CsA on IL-1α release:

3.3.1 FIBS cells:

100, 500 and 1000ng/ml had no significant effect on the release of IL-1α compared to control at all the time intervals studied (Fig 3.5). 2000ng/ml, however showed a significant increase ($p < 0.05$) between 8 and 24h (at 8h: 9.42 +/- 0.8 pg/ml vs control: 7.53 +/- 0.7 pg/ml; at 24h: 15.67 +/- 3.2 pg/ml vs control: 7.1 +/- 1.2 pg/ml), with levels returning to control values at the later time points.

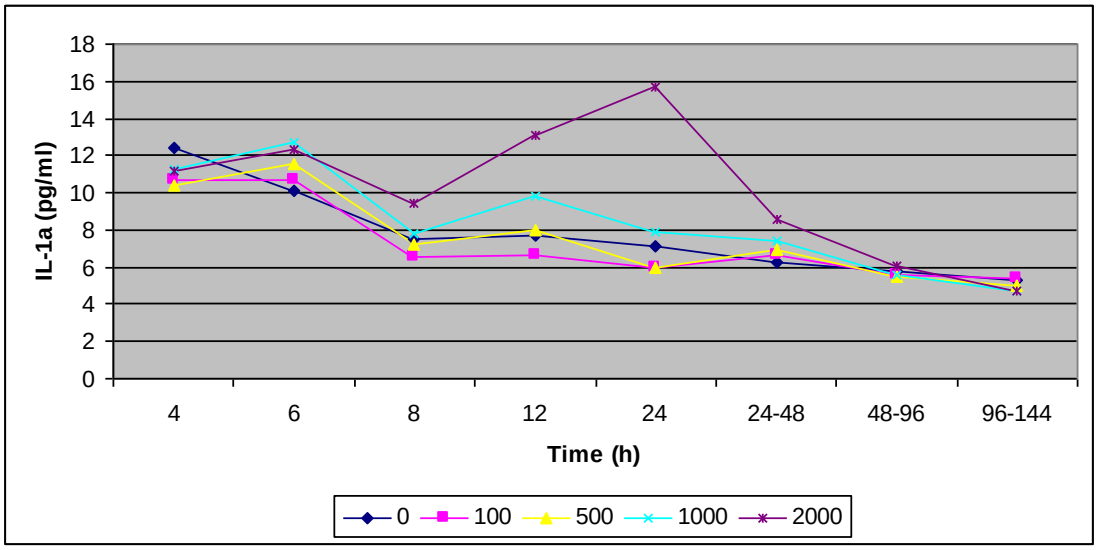

Figure. 3.5. Effect of CsA on IL-1α release by FIBS.
FIBS were stimulated with increasing doses of CsA and IL-1α was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.3.2 OKF6 cells:

100, 500 and 1000ng/ml had no significant effect on the release of IL-1α compared to control at all the time intervals studied (Fig 3.6). 2000ng/ml, however showed a significant increase ($p < 0.05$) between 24 and 48h (at 24h: 15.8 +/- 6.1 vs control: 7.5 +/- 1.3 pg/ml; at 48h: 11.8 +/- 0.5 vs control: 7.3 +/- 0.7 pg/ml), with levels returning to control values at the later time point.

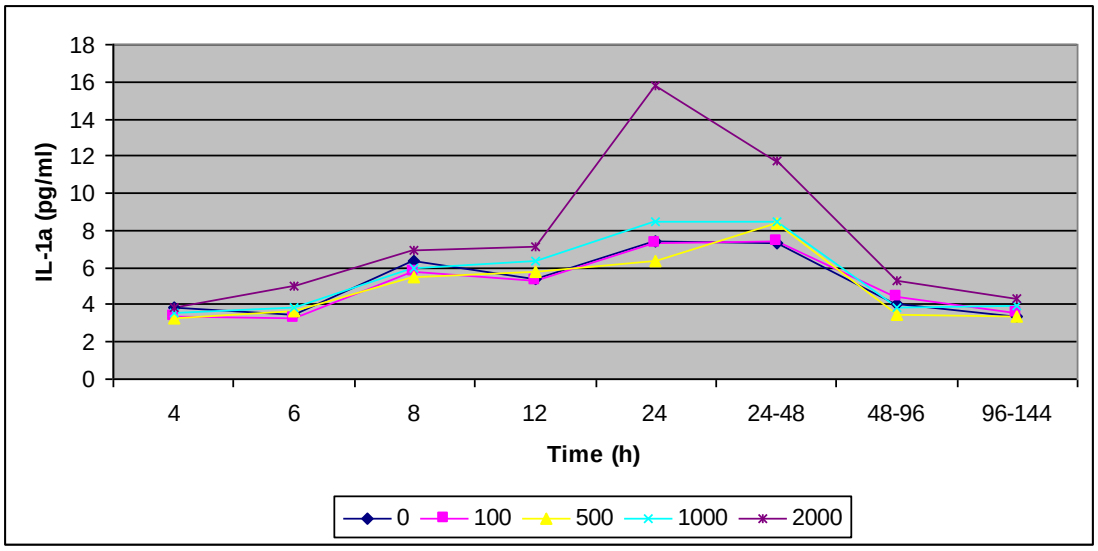

Figure 3.6. Effect of CsA on IL-1α release from OKF6.
OKF6 were stimulated with increasing doses of CsA and IL-1α was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.3.3 TR146 cells:

TR146 showed a dose dependent increase in IL-1α (Fig 3.7) with 2000ng/ml causing a maximum release after 24 hrs (13.5 +/- 1.2 vs control: 7.2 +/- 1 pg/ml). This effect was not evident at later time points.

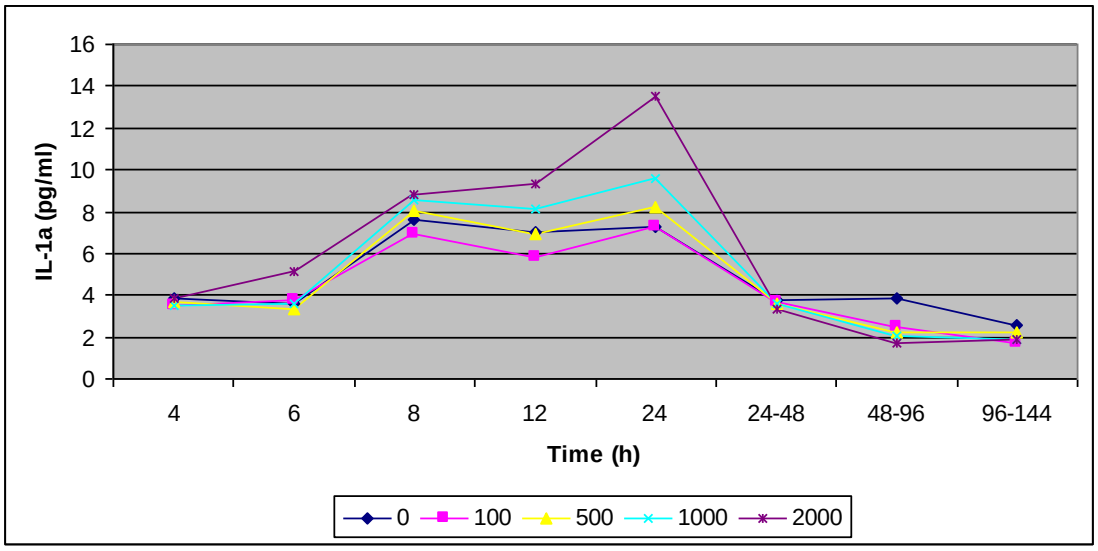

Figure. 3.7. Effect of CsA on IL-1α release from TR146.
TR146 were stimulated with increasing doses of CsA and IL-1α was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.3.4 HGF cells:

CsA had no significant effect on the release of IL-1α from HGF cells (Fig 3.8).

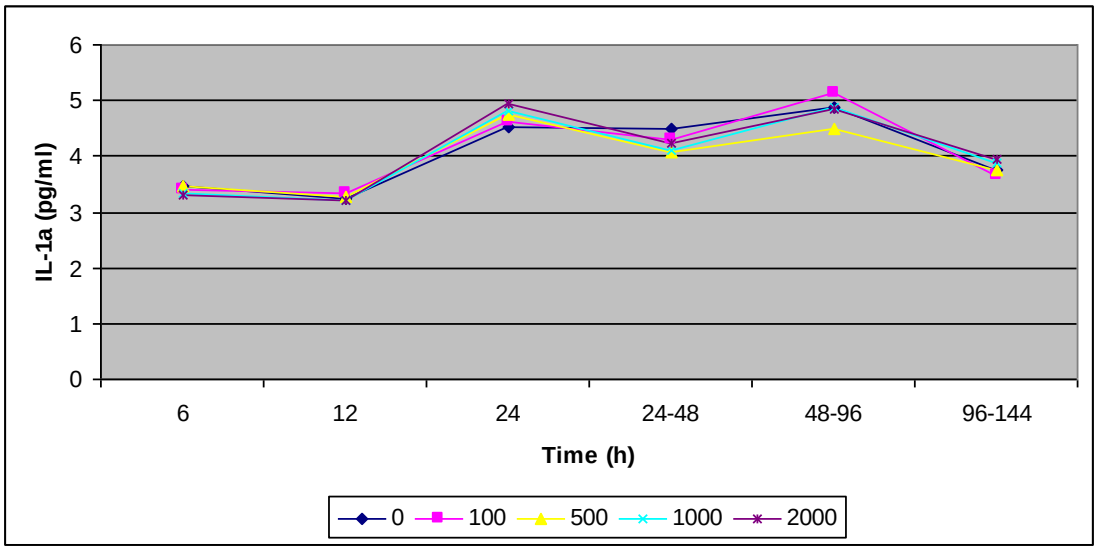

Figure 3.8 Effect of CsA on IL-1α release from HGF.
HGF were stimulated with increasing doses of CsA and IL-1α was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.4. Effect of CsA on IL-6 release:

3.4.1 FIBS cells:

Control cultures and those treated with 100, 500 and 1000ng/ml CsA showed a similar pattern with IL-6 release increasing up to 24hr (Fig 3.9). There was no significant difference between CsA treated and control cultures at these time points. 2000ng/ml CsA, however caused a significant increase ($p < 0.05$) in IL-6 release at 12 and 24 hr (at 12h: 349 +/- 92 vs control: 198 +/- 111 pg/ml; at 24h: 355 +/- 69 vs control: 266 +/- 61 pg/ml), returning to control values at the later time points

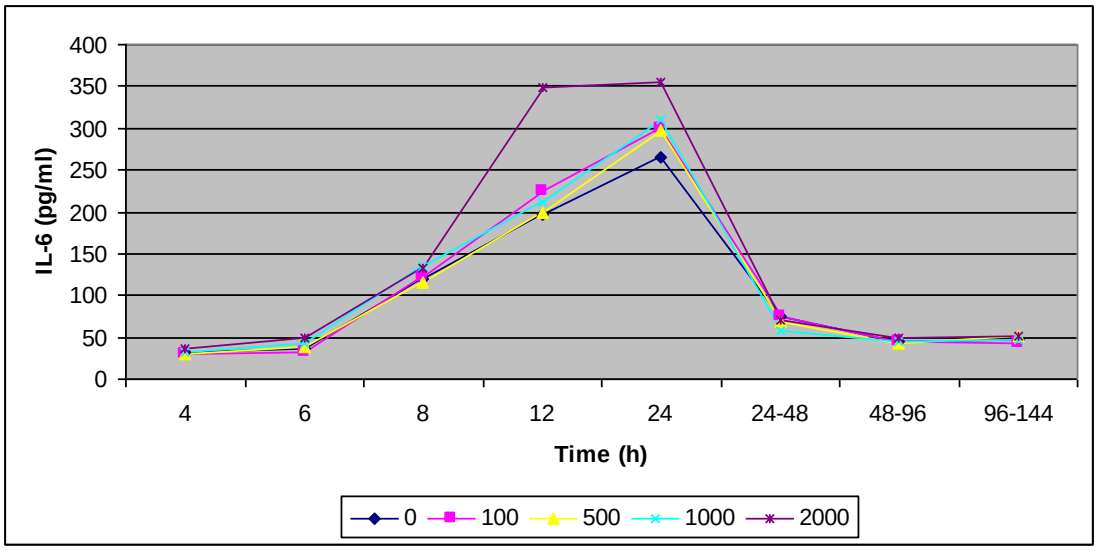

Figure 3.9 Effect of CsA on IL-6 release from FIBS.
FIBS were stimulated with increasing doses of CsA and IL-6 was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.4.2 OKF6 cells:

1000 and 2000ng/ml CsA caused a dose and time dependent increase in IL-6 release up to 24hr treatment (at 24h: 1000 ng/ml: 1023 +/- 246 and 2000ng/ml: 1355 +/- 271 ng/ml vs control: 529 +/- 92 pg/ml; $p < 0.05$; Fig 3.10). Levels of release returned to control values at the later time points. 100 and 500ng/ml had no significant effect on IL-6.

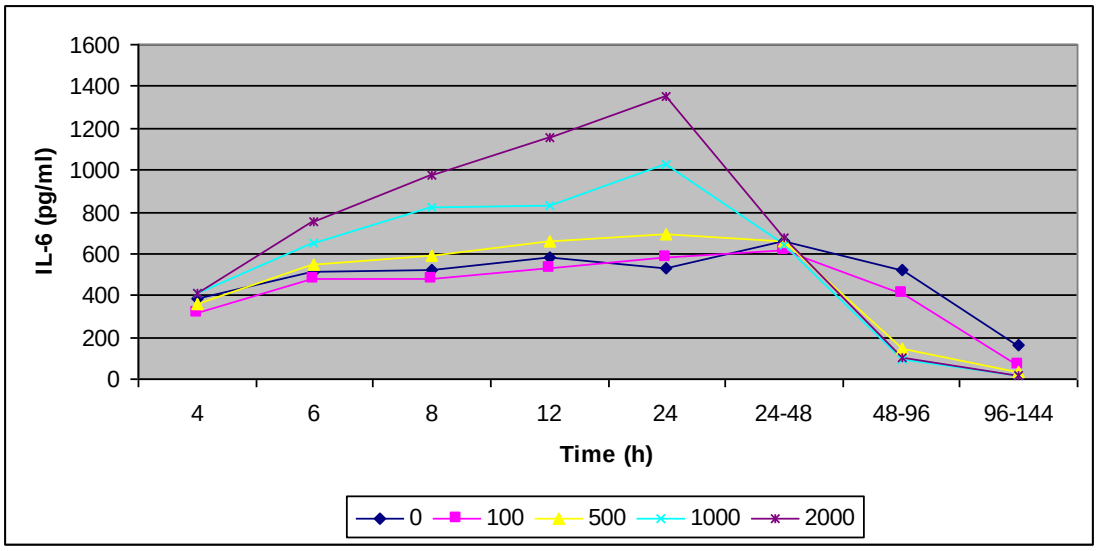

Figure 3.10 Effect of CsA on IL-6 release from OKF6.
OKF6 were stimulated with increasing doses of CsA and IL-6 was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.4.3 TR146 cells:

500. 1000 and 2000ng/ml CsA showed a maximum release of IL-6 after 12 h (424 +/- 52, 462 +/- 73, and 565 +/- 130 pg/ml respectively vs control: 302 +/- 48 pg/ml; $p < 0.05$; Fig 3.11) which returned to control levels by 24h (500 and 1000ng/ml) or 48h (2000ng/ml). 100ng/ml had no significant effect on IL-6 release.

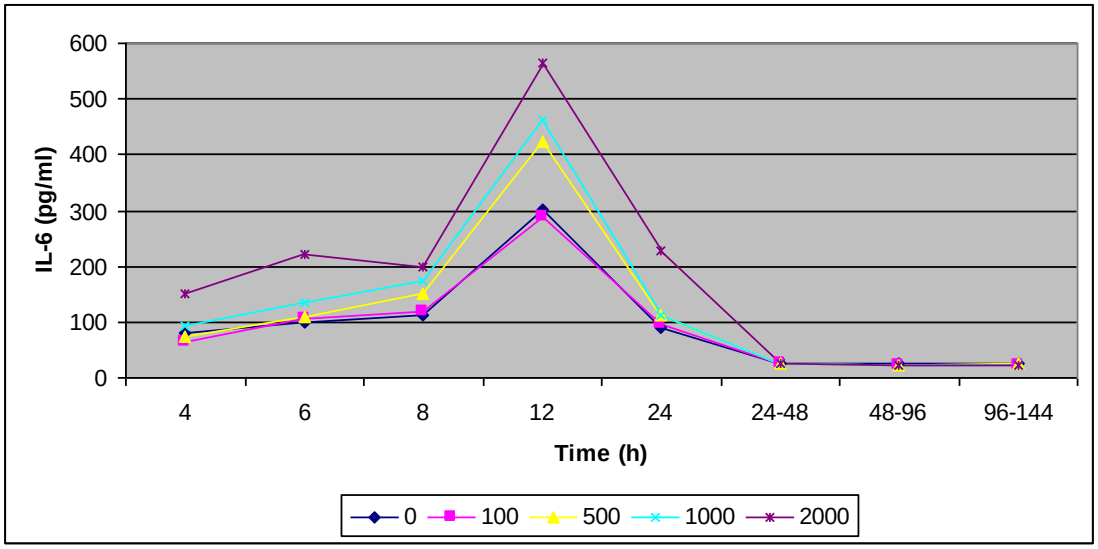

Figure 3.11 Effect of CsA on IL-6 release from TR146.
TR146 were stimulated with increasing doses of CsA and IL-6 was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.4.4 HGF cells:

CsA had no significant effect on IL-6 release in HGF at any point of the treatment time course ($p < 0.05$ Fig 3.12).

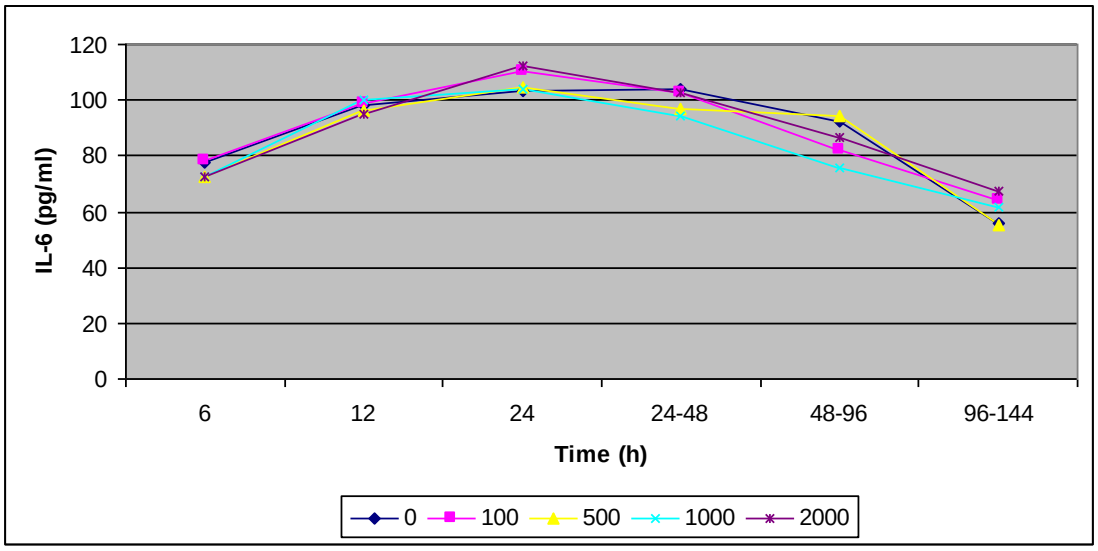

Figure 3.12 Effect of CsA on IL-6 release from HGF.
HGF were stimulated with increasing doses of CsA and IL-6 was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.5. Effect of CsA on IL-8 release:

3.5.1 FIBS cells:

500, 1000 and 2000ng/ml CsA showed slight but significant increases in IL-8 release up to 48h (at 24h: 1160 +/- 118, 1196 +/- 50, and 1202 +/- 98 pg/ml respectively vs control: 1016 +/- 120 pg/ml; $p < 0.05$; Fig 3.13). This increase was maintained up to 144hr with 2000ng/ml CsA only (932 +/- 153 and 967 +/- 29 pg/ml at 96h and 144h respectively vs control: 445 +/- 122, and 69 +/- 27 pg/ml).

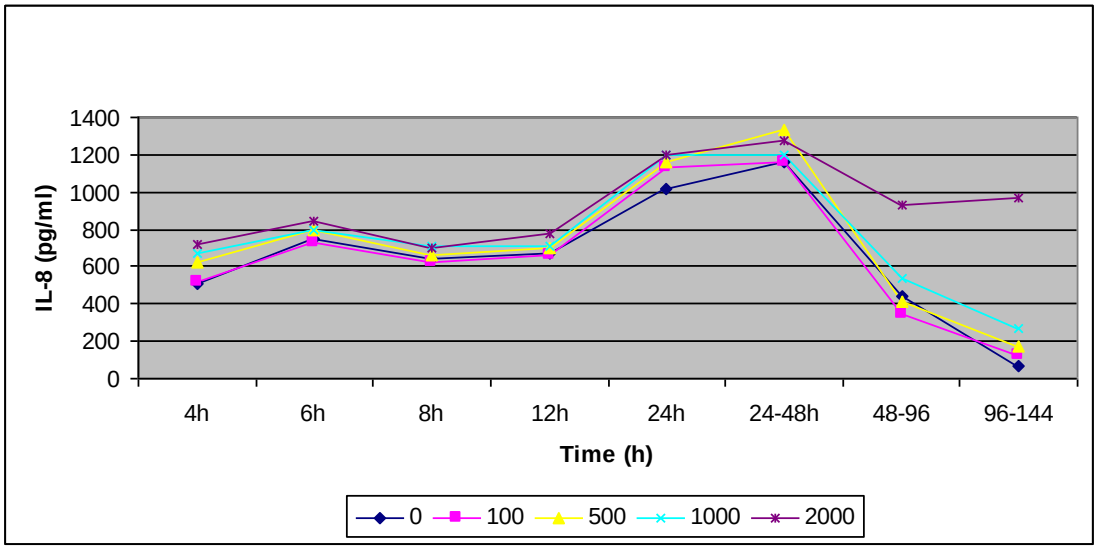

Figure 3.13 Effect of CsA on IL-8 release from FIBS.
FIBS were stimulated with increasing doses of CsA and IL-8 was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity.

3.5.2 OKF6 cells:

2000ng/ml CsA caused a slight but significant increase in IL-8 release up to 24h (625 +/- 63, 740 +/- 28, 818 +/- 47, 882 +/- 91, and 1110 +/- 120 at 4, 6, 8, 12, and 24h respectively vs control: 463 +/- 40, 624 +/- 28, 691 +/- 104, 742 +/- 50, and 985 +/- 138 pg/ml; $p<0.05$; Fig 3.14) but had no significant effect afterwards. Lower doses of CsA had a very little effect.

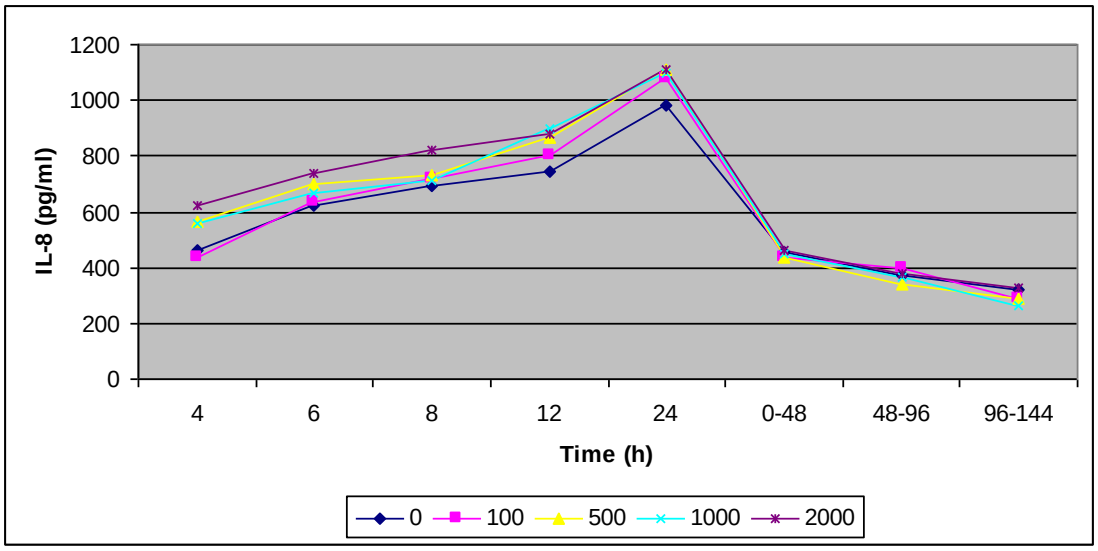

Figure 3.14 Effect of CsA on IL-8 release from OKF6.
OKF6 were stimulated with increasing doses of CsA and IL-8 was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity

3.5.3 TR146 cells:

TR146 showed a maximal IL-8 release after 12 exposure to 500, 1000 and 2000ng/ml CsA (88 +/- 31, 195 +/- 82, and 364 +/- 40 pg/ml respectively vs control: 42 +/- 25 pg/ml; $p < 0.05$) returning to control levels by 24 – 48 h (Fig 3.15).

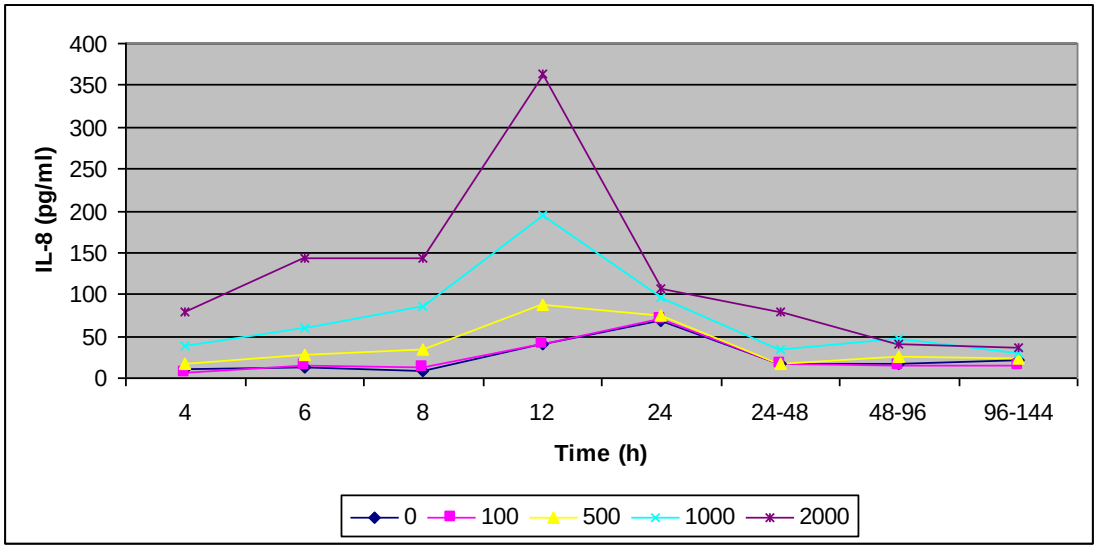

Figure 3.15 Effect of CsA on IL-8 release from TR146.
TR146 were stimulated with increasing doses of CsA and IL-8 was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity

3.5.4 HGF cells:

CsA had no significant effect on the release of IL-8 from HGF cells (Fig 3.16)

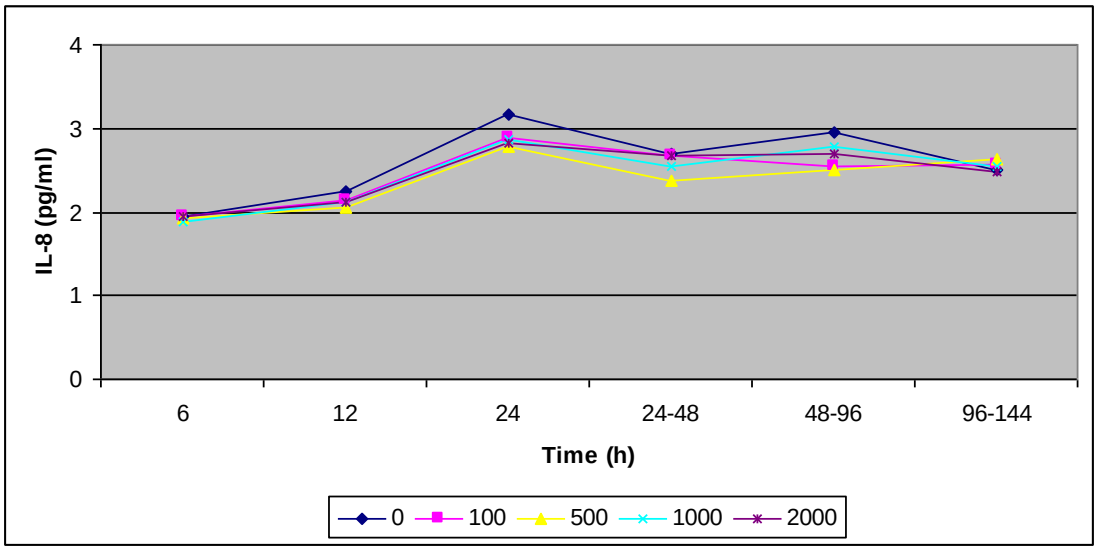

Figure 3.16 Effect of CsA on IL-8 release from HGF.
HGF were stimulated with increasing doses of CsA and IL-8 was measured by ELISA. Control cultures were stimulated with Ethanol vehicle (1/1000 in DMEM); n = 9. SEM has been omitted for clarity

3.6 Summary and Conclusions

Viability

All doses of CsA tested had little or no effect on the viability of FIBS, TR146 and HGF cells up to 144h exposure. OKF6 cells, however only showed a significant reduction in viability after prolonged exposure (up to 144hr) to higher doses (1000 and 2000ng/ml).

Cytokine release

Overall CsA had a variable effect on the release of IL-1α, IL-6 and IL-8 from the FIBS, OKF6, TR146 and HGF. 1000 and 2000ng/ml stimulated cytokine release from the keratinocytes after 12 – 24 h exposure whilst HGF cells were not affected by CsA. Prolonged exposure did not demonstrate a significant change in cytokine release. As a result 2000ng/ml CsA for 24h was chosen for the next phase of the study with 250ng/ml as the low concentration that reflects in vivo measurements (Fisher *et al.*, 1988).

Chapter Four

Effect of CsA and bacterial supernatants on cell viability and cytokine expression in monolayer cell cultures

4.1. Introduction:

The results outlined in the previous chapter demonstrated that CsA had a limited effect on cell viability and that cytokine release was maximal after 24h exposure with the highest dose. However, *in vivo* any interaction between CsA and host cells will usually take place in the presence of dental plaque or plaque derived products. The aim of the next phase of the study, therefore was to examine the effect of the combination of CsA (2000ng/ml CsA for 24h) and four periodontopathic organisms (P.g, A.a, P.i, and F.n) on viability and cytokine release. In addition, the effect of 250ng/ml CsA was also tested as this reflects in vivo measurements (Fisher *et al.*, 1988). The cell viability and cytokine release were determined by MTT assay and ELISA respectively. The significant effect of each combined treatment of (CsA and bacterial supernatant) was determined by comparing to three controls: control cultures, CsA alone, and the respective bacterial supernatant alone using one-way ANOVA together with Student's independent t-test.

4.2. Determination of the bacterial dose:

Toxicity of bacterial supernatants was determined by evaluating the integrity of cell membrane of the studied cell lines after 24h of bacterial challenge through measuring the expression level of Lactate Dehydrogenase LDH in the culture medium using an *in vitro* Cytotoxicity Detection Kit^{plus} (LDH) (Roche, Mannheim, Germany). The results were confirmed by measuring the effect of bacterial supernatants on the viability of cell lines by Alamar blue (Invitrogen.UK).

Bacterial supernatants at 1:10 and 1:50 had a greater effect on LDH release and cell viability than 1:100 in FIBS (Fig 4.1 & 4.2). A similar result was obtained for the other cell lines (Appendix 1). Bacterial supernatant at 1:100 was therefore chosen for the subsequent experiments

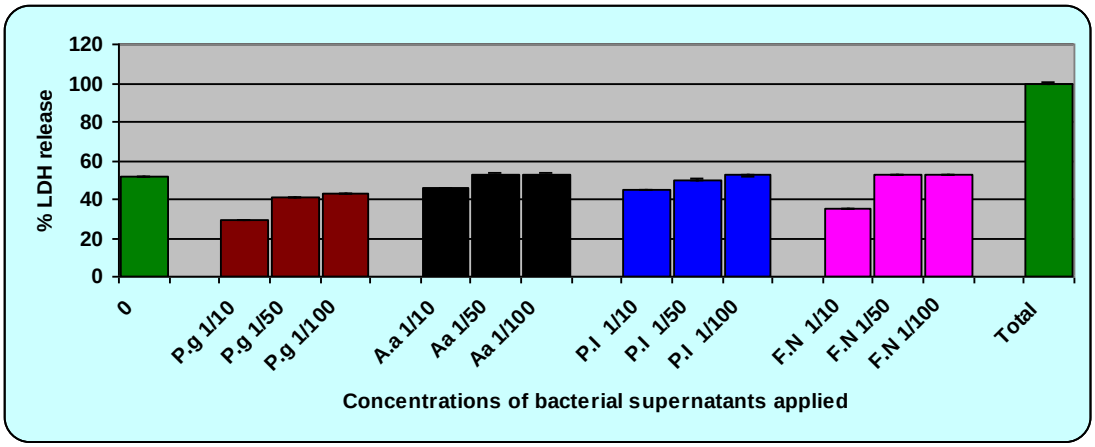

Figure 4.1. LDH release from FIBS. Cell lines were challenged for 24h with ascending doses of bacterial supernatants. Extracellular release of LDH was then measured using a Cytotoxicity Detection Kit^{plus}. Two controls were used in the study; high control which represents the totally lysed cells and low control which is medium containing un-treated cells. Results are expressed as percentage of total LDH. Bars represent Mean ± SEM of 12 samples.

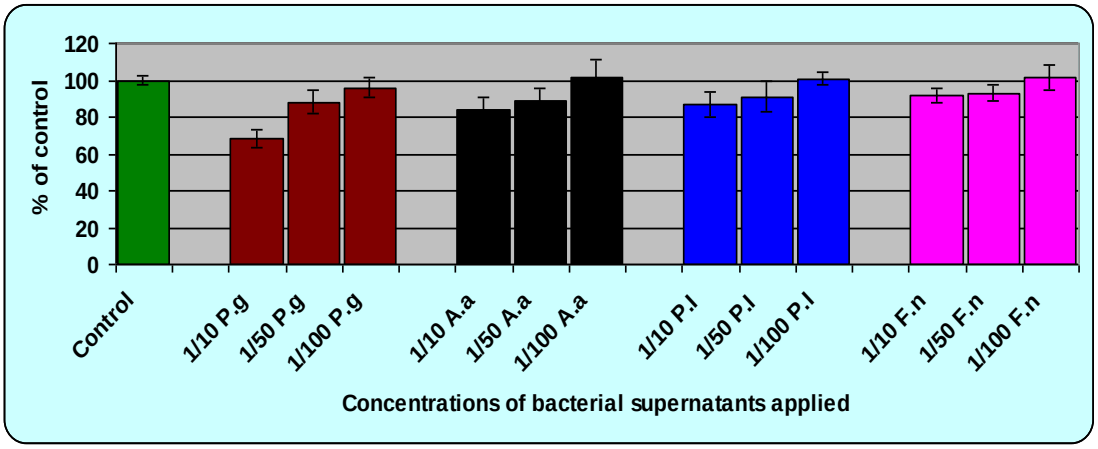

Figure 4.2. FIBS viability. Cultures were stimulated for 24h with ascending concentrations of bacterial supernatant. Cell viability was measured by Alamar blue. Untreated cells were served as control. Results are expressed as the Mean ± SEM (n=9). Statistical significance was regarded when $p < 0.05$ compared to control.

4.3. Treatment:

Cells were seeded into 24 well plates and allowed to settle down for 24h. Cells were washed twice with PBS and then serum-starved for 24h.

After washing with PBS, cells were pre-stimulated for 24h with bacterial supernatants (either 0 or 1:100 dilutions of P.g, A.a, F.n, and P.i in growth medium) at 37^{o}C and 5% CO_2. Cells were washed with PBS and then stimulated for additional 24h with different concentrations of CsA (2000ng/ml, 250 ng/ml and 0ng/ml as a control). At the end of the experiment, culture supernatants were collected and frozen at -80^{o}C until use for cytokine analysis and MTT assay was conducted to measure the cell viability. Controls included un-treated cultures, bacterial supernatant-treated cultures without CsA, and cultures treated with CsA only.

4.4. Effect of CsA and bacteria supernatants on cell viability:

4.4.1. FIBS cells:

250ng/ml CsA caused a slight increase in the FIBS cell viability compared to the control (250ng/ml CsA; 114 ± 12% vs control; 100 ± 4%. Fig 4.3; $p<0.05$). Conversely, 2000ng/ml CsA caused a slight decrease in the FIBS cell viability (2000ng/ml; 92 ± 7%; $p<0.05$)

P. gingivalis

P.g significantly decreased the viability of FIBS cells compared to the control (P.g; 86 ± 10%; $p<0.05$ vs control). Addition of CsA significantly promoted this inhibitory effect on FIBS viability (P.g + 250ng/ml CsA; 72 ± 3, P.g + 2000ng/ml CsA; 71 ± 4% $p<0.05$ vs P.g)

A. actinomycetemcomitans

A.a significantly increased the viability of FIBS cells compared to the control (A.a; 109 ± 9%; $p<0.05$ vs control). Addition of 250ng/ml CsA did not cause a significant modulation on FIBS viability. 2000ng/ml CsA, however abrogated the effect of A.a on cell viability with results returning to control levels (A.a + 2000ng/ml CsA; 93 ± 10%; $p<0.05$ vs A.a).

P. intermedia

P.i significantly increased the viability of FIBS cells compared to that of control (P.i; 147 ± 16%; $p<0.05$ vs control). Furthermore, addition of either 250ng/ml or 2000ng/ml CsA significantly decreased the cell viability with results returning to levels found in cultures treated with 250ng/ml and 2000ng/ml CsA respectively (P.i + 250ng/ml CsA; 118 ± 10, P.i + 2000ng/ml; 102 ± 16%; $p<0.05$ vs P.i).

F. nucleatum

F.n significantly increased the viability of FIBS cells compared to that of control (F.n; 124 ± 11%; $p<0.05$ vs control). Addition of 250ng/ml CsA significantly promoted this stimulatory effect on FIBS cell viability (F.n + 250ng/ml CsA; 146 ± 22%; $p<0.05$ vs F.n). Conversely, addition of 2000ng/ml CsA abrogated the effect of F.n on cell viability with results returning to control levels (F.n + 2000ng/ml CsA; 102 ± 13%; $p<0.05$ vs F.n)

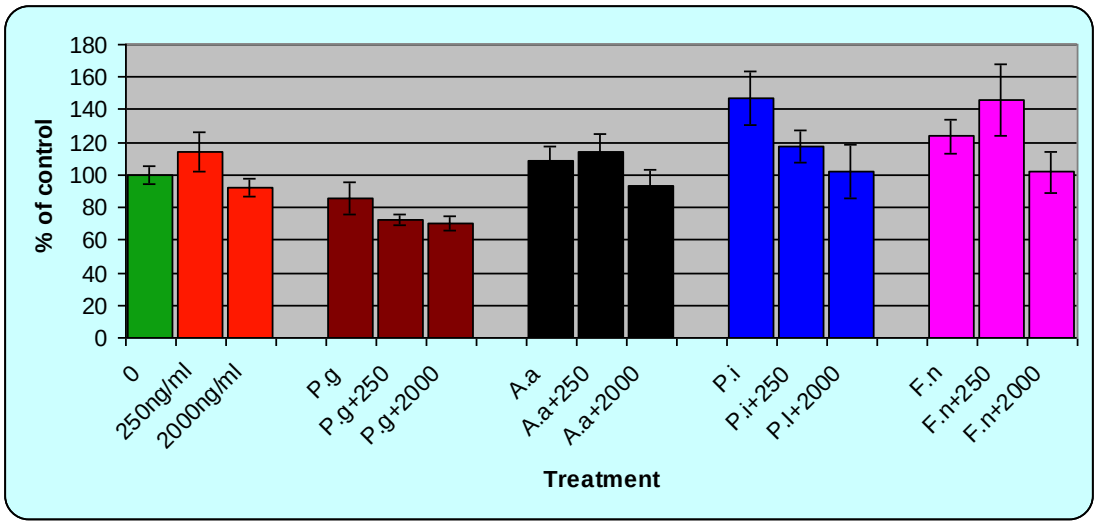

Figure 4.3. Effect of (CsA + bacterial supernatant) combination on FIBS viability. Cultures were stimulated with CsA or/and bacterial supernatant. Cell viability was measured by MTT assay after 24h exposure. Untreated cells were served as a control. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p < 0.05$ compared to control.

4.4.2 OKF6 cells:

CsA at either 250ng/ml or 2000ng/ml dose caused a significant increase in the cell viability compared to the control (250ng/ml; 121 ± 10, 2000ng/ml CsA; 132 ± 14 vs control; 100 ± 9%; Fig 4.4; $p<0.05$).

P. gingivalis

P.g significantly increased the viability of OKF6 cells compared to that of control (P.g; 144 ± 23%; $p<0.05$ vs control). Furthermore, addition of CsA had no significant effect on the cell viability (P.g + 250ng/ml CsA; 137 ± 24, P.g + 2000ng/ml CsA; 132 ± 21%; $p>0.05$ vs P.g).

A. actinomycetemcomitans

A.a significantly decreased the viability of OKF6 cells compared to that of control (A.a; 81 ± 11%; $p<0.05$ vs control). However, addition of 250ng/ml CsA significantly increased the cell viability with values returning to control level (A.a + 250ng/ml CsA; 105 ± 20%; $p<0.05$ vs A.a). Conversely, addition of 2000ng/ml CsA had no effect on OKF6 viability (A.a + 2000ng/ml CsA; 81 ± 13%; $p>0.05$ vs A.a).

P. intermedia

P.i significantly increased the viability of OKF6 cells compared to that of controls (P.i; 158 ± 34%; $p<0.05$ vs control). However, addition of CsA had no significant effect on the cell viability ($p>0.05$ vs P.i).

F. nucleatum

F.n supernatant significantly increased the viability of OKF6 cells compared to that of control (F.n; 125 ± 34%; $p<0.05$ vs control). Furthermore, addition of CsA had no significant effect on the cell viability ($p>0.05$ vs F.n).

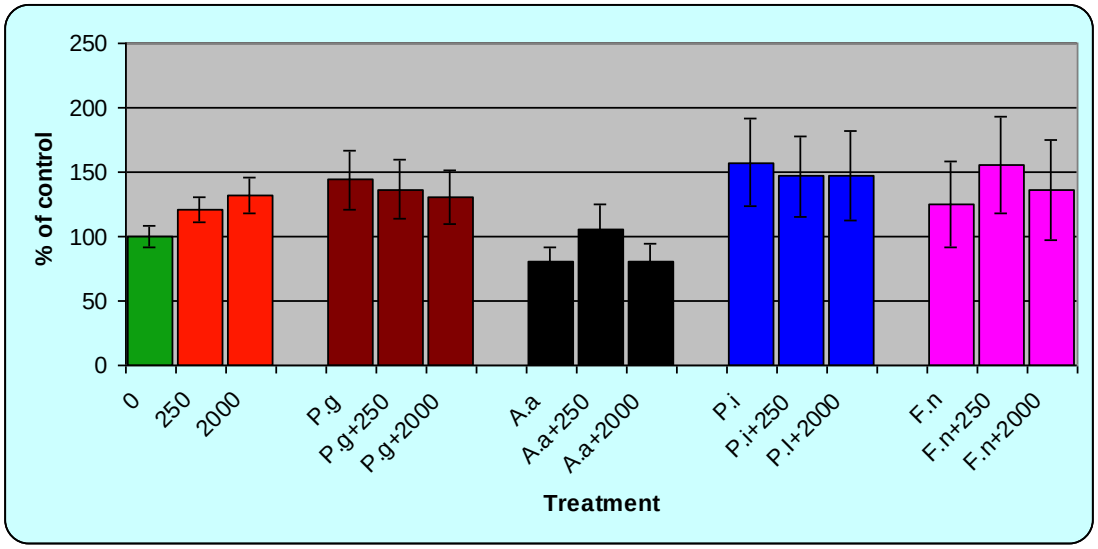

Figure 4.4. Effect of (CsA + bacterial supernatant) combination on OKF6 viability. Cultures were stimulated with CsA or/and bacterial supernatant. Cell viability was measured by MTT assay after 24h exposure. Untreated cells were served as a control. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p< 0.05$ compared to control.

4.4.3 TR146 cells:

CsA at either 250ng/ml or 2000ng/ml dose had no significant effect on TR146 viability (107 ± 9 & 98 ± 11 respectively vs control; 100 ± 7%; Fig 4.5; $p<0.05$).

P. gingivalis

P.g significantly increased the viability of TR146 compared to the controls (P.g; 186 ± 21%; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Addition of

250ng/ml CsA did not cause a significant modulation on the cell viability. However, addition of 2000ng/ml CsA significantly decreased the cell viability (P.g + 2000ng/ml CsA; 131 ± 15%; $p<0.05$ vs P.g) with values still significantly higher than that of control and cultures treated with 2000ng/ml CsA ($p<0.05$ vs control and 2000ng/ml CsA).

A. actinomycetemcomitans

A.a significantly increased the viability of TR146 cells compared to the controls (A.a; 125 ± 24%; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Furthermore, addition of 250ng/ml CsA significantly enhanced this stimulatory effect (A.a + 250ng/ml CsA; 187 ± 30%; $p<0.05$ vs A.a). However, addition of 2000ng/ml CsA had no effect.

P. intermedia

P.i significantly increased the viability of TR146 cells compared to the controls (P.i; 193 ± 27%; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Addition of 250ng/ml CsA had no significant effect on the cell viability. However, addition of 2000ng/ml CsA significantly reduced the cell viability (P.i + 2000ng/ml CsA; 156 ± 17%; $p<0.05$ vs P.i) with values still significantly higher than that of control and cultures treated with 2000ng/ml CsA ($p<0.05$ vs control and 2000ng/ml CsA).

F. nucleatum

F.n significantly increased the viability of TR146 cells compared to the controls (F.n; 136 ± 16%; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Addition of 250ng/ml CsA significantly promoted this stimulatory effect on cell viability (F.n + 250ng/ml CsA; 194 ± 27%; $p<0.05$ vs F.n). However, addition of 2000ng/ml CsA had no effect.

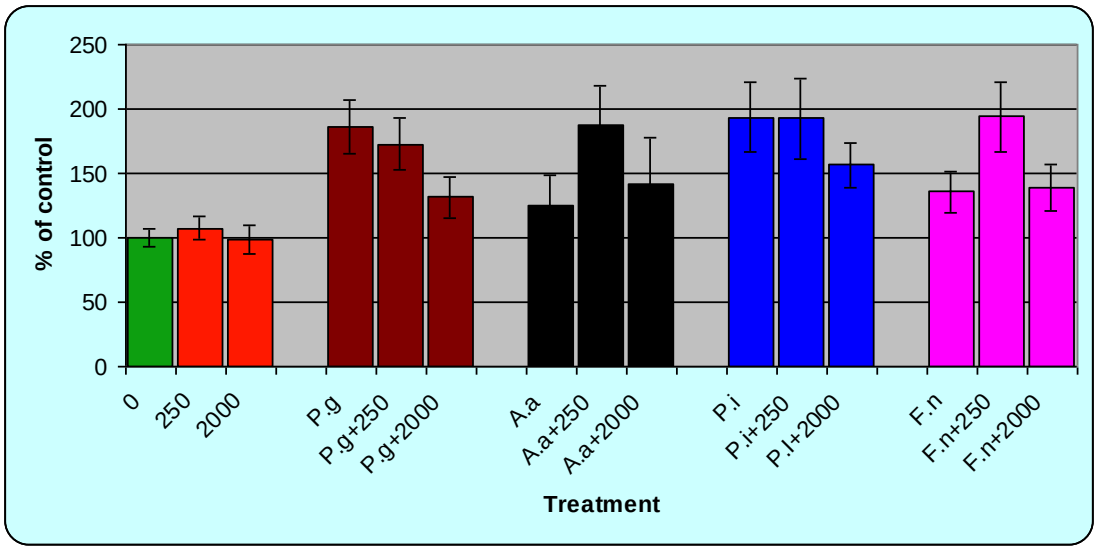

Figure 4.5. Effect of (CsA + bacterial supernatant) combination on TR146 viability. Cultures were stimulated with CsA or/and bacterial supernatant. Cell viability was measured by MTT assay after 24h exposure. Untreated cells were served as a control. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p < 0.05$ compared to control.

4.4.4 HGF cells:

250ng/ml CsA had no significant effect on cell viability (104 ± 12 vs control; 100 ± 6%; $p>0.05$). However, 2000ng/ml CsA caused a significant increase in the HGF viability compared to the control (114 ± 15%; Fig 4.6; $p<0.05$ vs control).

P. gingivalis

P.g had no significant effect on HGF viability compared to the control (92 ± 12%; $p>0.05$ vs control). Addition of CsA at (250ng/ml or 2000ng/ml) had no significant effect on the cell viability ($p>0.05$ vs P.g).

A. actinomycetemcomitans

A.a significantly decreased the cell viability compared to the controls (86 ± 12%; $p<0.05$ vs control). Addition of 250ng/ml CsA did not cause a significant modulation on the cell viability ($p>0.05$ vs A.a). However, addition of 2000ng/ml CsA significantly increased the cell viability (A.a + 2000ng/ml; 104 ± 14%; $p<0.05$ vs A.a) with values returning to control levels ($p>0.05$ vs control & 2000ng/ml).

P. intermedia

Stimulation of HGF with P.i supernatant either individually or in combination with CsA has no significant effect on HGF viability.

F. nucleatum

F.n supernatant significantly decreased the cell viability compared to controls (81 ± 19%; $p<0.05$ vs controls). However, addition of CsA had no significant effect on cell viability ($p>0.05$ vs F.n).

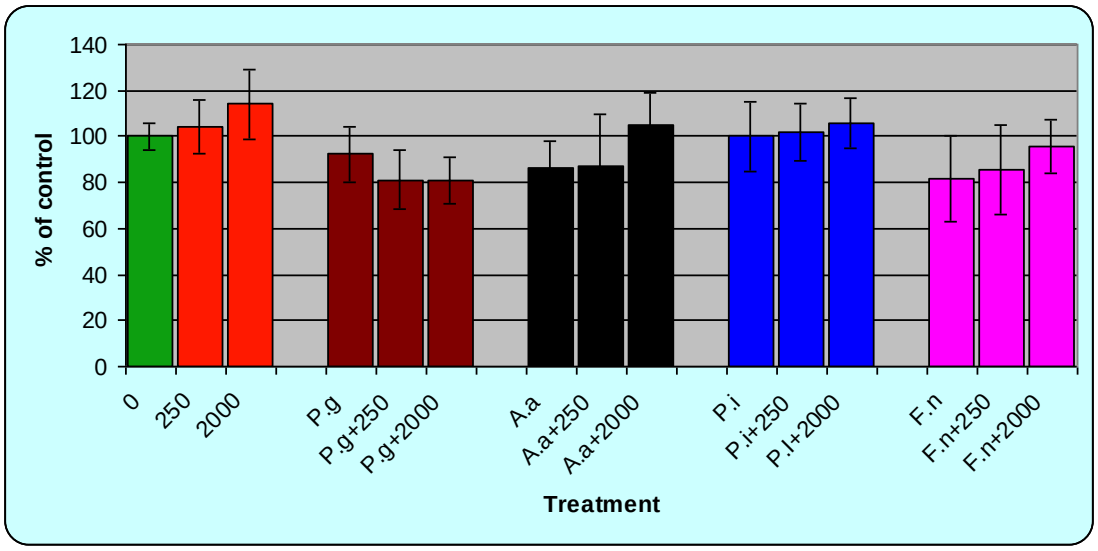

Figure 4.6. Effect of (CsA + bacterial supernatant) combination on HGF viability. Cultures were stimulated with CsA or/and bacterial supernatant. Cell viability was measured by MTT assay after 24h exposure. Untreated cells were served as a control. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p< 0.05$ compared to control.

4.5. Effect of CsA and bacteria supernatants on IL-1α release:

4.5.1 FIBS cells:

250ng/ml CsA did not cause a significant modulation on IL-1α release compared to the control (250ng/ml CsA; 17 ± 5 vs control; 15 ± 4pg/ml; $p>0.05$). Furthermore, 2000ng/ml CsA significantly increased IL-1α release (2000ng/ml CsA; 29 ± 7pg/ml; Fig 4.7; $p<0.05$ vs control).

P. gingivalis

P.g had no significant effect on IL-1α release compared to that of the control (P.g; 18 ± 3pg/ml; $p>0.05$ vs control & 250ng/ml CsA). Addition of 250ng/ml CsA did not significantly modulate IL-1α release. However, addition of 2000ng/ml CsA significantly increased IL-1α release with values returning to that level being found in cultures treated with 2000ng/ml CsA (P.g + 2000ng/ml CsA; 28 ± 6pg/ml; $p<0.05$ vs P.g).

A. actinomycetemcomitans

A.a had no significant effect on IL-1α release compared to that of the control (A.a; 16 ± 3pg/ml; $p>0.05$ vs control & 250ng/ml CsA). Addition of 250ng/ml CsA did not significantly modulate the release of IL-1α. However, addition of 2000ng/ml CsA significantly increased IL-1α release with values still significantly lower than that of cultures treated with 2000ng/ml CsA (A.a + 2000ng/ml CsA; 21 ± 3pg/ml; $p<0.05$ vs A.a & 2000ng/ml CsA individually).

P. intermedia

P.i significantly decreased IL-1α release compared to that of controls (P.i; 10 ± 1pg/ml; $p<0.05$ vs control and CsA 250ng/ml & 2000ng/ml). Addition of 250ng/ml CsA did not significantly modulate IL-1α release. However, addition of 2000ng/ml CsA significantly increased release of IL-1α with values still significantly lower than that of cultures treated with 2000ng/ml CsA(P.i + 2000ng/ml CsA; 19 ± 3pg/ml; $p<0.05$ vs P.i & 2000ng/ml CsA individually).

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F. nucleatum

F.n caused a similar effect on IL-1α release to that found with P.i either individually or in combination with CsA.

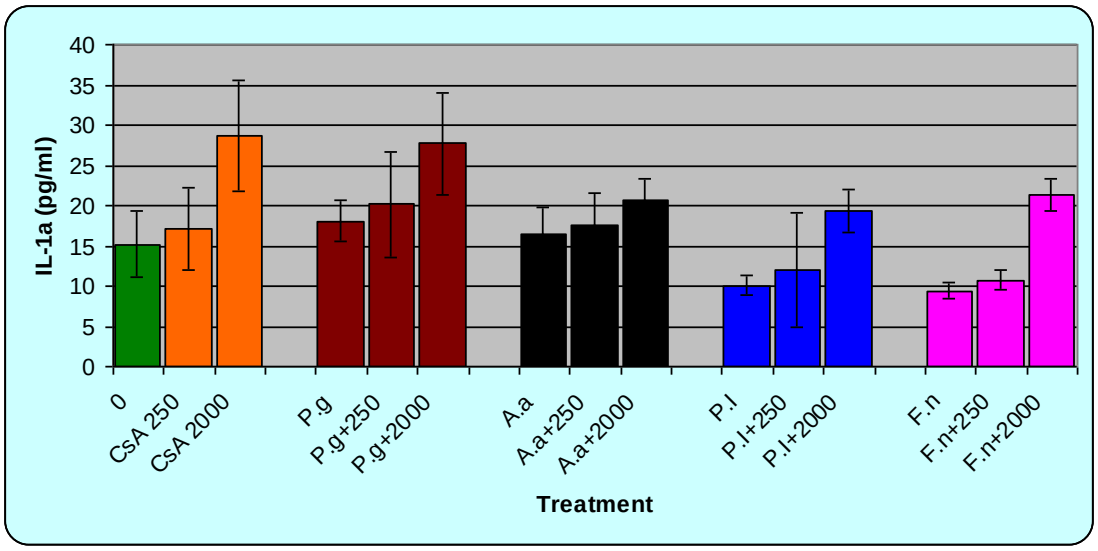


Figure 4.7. Effect of (CsA + bacterial supernatant) combination on release of IL-1α in FIBS. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-1α was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p < 0.05$ compared to control.

4.5.2 OKF6 cells:

250ng/ml CsA did not cause a significant modulation on IL-1α release compared to the control (250ng/ml CsA; 5 ± 0.3 vs control 5 ± 0.3pg/ml; $p>0.05$). Furthermore, 2000ng/ml CsA significantly increased IL-1α release compared to control (2000ng/ml CsA; 8 ± 0.4pg/ml; Fig 4.8; $p<0.05$ vs control).

P. gingivalis

P.g significantly decreased IL-1α release compared to that of controls (P.g; 3 ± 0.2pg/ml; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Addition of 250ng/ml CsA significantly increased IL-1α release with values returning to that level being found in control culture and cultures treated with 250ng/ml CsA (P.g + 250ng/ml CsA; 4.6 ± 0.8pg/ml; $p<0.05$ vs P.g). However, addition of 2000ng/ml CsA had no significant effect.

A. actinomycetemcomitans

A.a significantly increased IL-1α release compared to that of controls (A.a; 22 ± 3.5pg/ml; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Furthermore, addition of either 250ng/ml or 2000ng/ml CsA had no significant effect on IL-1α release ($p>0.05$ vs A.a).

P. intermedia and F. nucleatum

P.i and F.n caused a significant decrease in IL-1α release compared to that of controls (2.5 ± 0.1 & 2.6 ± 0.2pg/ml respectively; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). However, addition of 250ng/ml or 2000ng/ml CsA had no significant effect on IL-1α release ($p>0.05$ vs the respective bacterial supernatant).

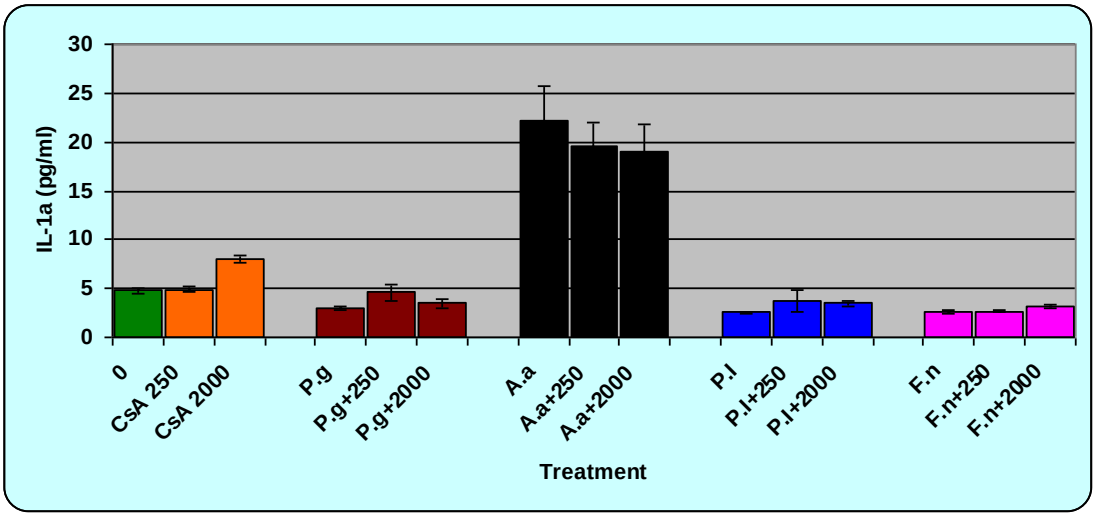


Figure 4.8. Effect of (CsA + bacterial supernatant) combination on release of IL-1α in OKF6. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-1α was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p< 0.05$ compared to control.

4.5.3 TR146 cells:

250ng/ml CsA did not cause a significant modulation on IL-1α release compared to the control (250ng/ml CsA; 9.6 ± 5.5 vs control; 6.6 ± 1.2pg/ml; $p>0.05$). However, 2000ng/ml CsA significantly increased the release of IL-1α (2000ng/ml CsA; 14.1 ± 4pg/ml; Fig 4.9; $p<0.05$ vs control).

P. gingivalis

P.g significantly increased IL-1α release compared to the control (P.g; 9.7 ± 3.3pg/ml; $p<0.05$ vs control). Addition of either 250ng/ml or 2000ng/ml CsA had no significant effect on IL-1α release.

A. actinomycetemcomitans

A.a had no significant effect on IL-1α release compared to the control (A.a; 6.9 ± 0.8pg/ml; $p>0.05$ vs control) Addition of 250ng/ml CsA had no significant effect. However, addition of 2000ng/ml CsA significantly increased the release of IL-1α with values still significantly lower than that of cultures treated with 2000ng/nl CsA only (A.a + 2000ng/ml CsA; 10.3 ± 2pg/ml; $p<0.05$ vs A.a & 2000ng/ml CsA individually).

P. intermedia

P.i significantly increased IL-1α release compared to the control (P.i; 10.3 ± 2.5pg/ml; $p<0.05$ vs control). Addition of 250ng/ml CsA did not significantly modulate the release of IL-1α. However, addition of 2000ng/ml CsA significantly increased IL-1α release with values returning to that level found in cultures treated with 2000ng/ml CsA (P.i + 2000ng/ml CsA; 16 ± 3.5pg/ml; $p<0.05$ vs P.i).

F. nucleatum

F.n did not cause a significant modulation on IL-1α release compared to the control (F.n; 7.4 ± 2.4pg/ml; $p>0.05$ vs control). Addition of 250ng/ml CsA had no significant effect. However, addition of 2000ng/ml CsA significantly increased the release of IL-1α with values returning to that level found in cultures treated with 2000ng/ml CsA (F.n + 2000ng/ml CsA; 17.3 ± 2.6pg/ml; $p<0.05$ vs F.n).

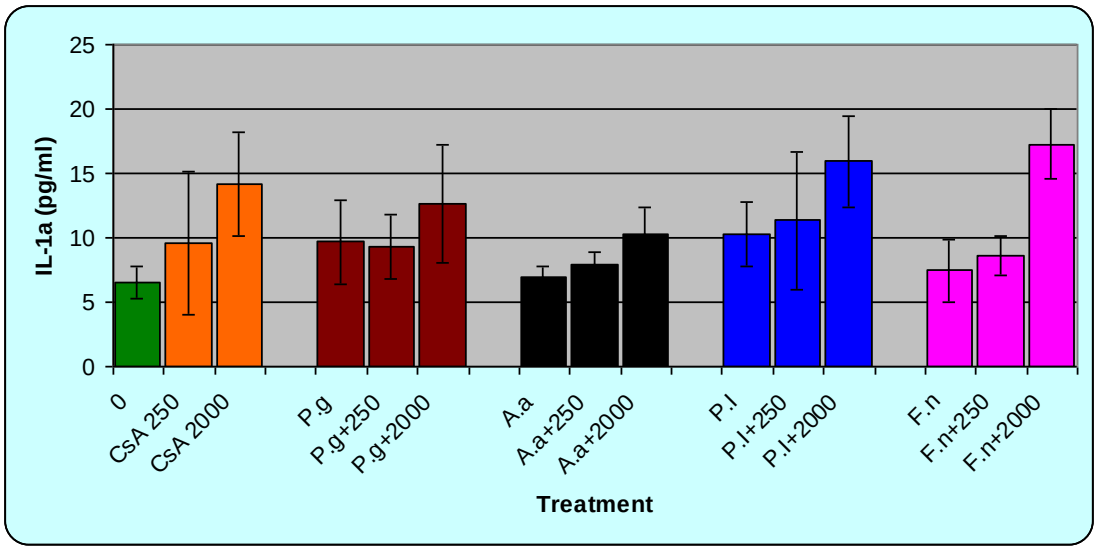


Figure 4.9. Effect of (CsA + bacterial supernatant) combination on release of IL-1α in TR146. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-1α was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p < 0.05$ compared to control.

4.5.4 HGF cells:

CsA and bacterial supernatants had no significant effect on the release of IL-1α from HGF cells (Fig 4.10)

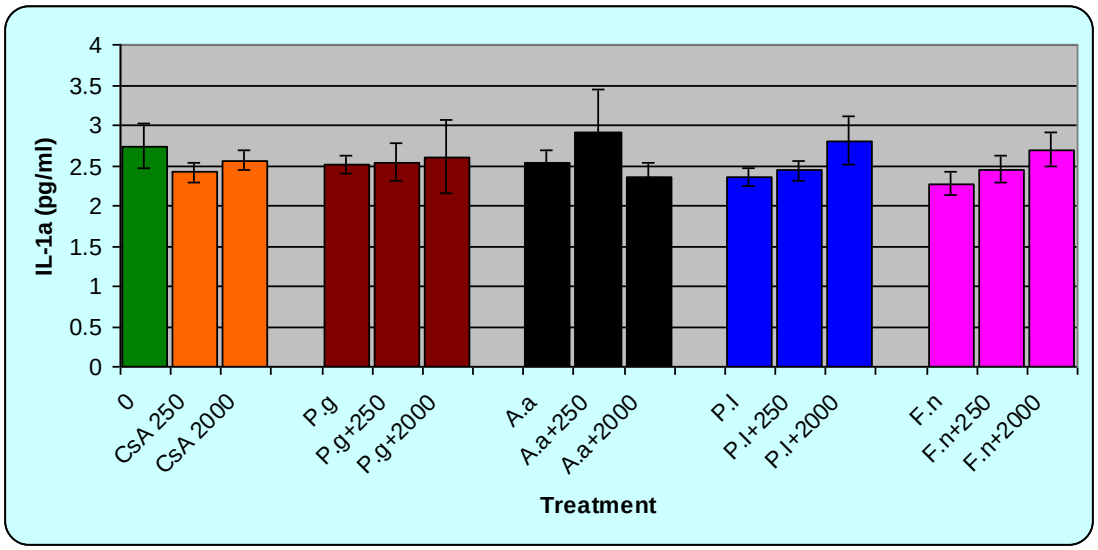


Figure 4.10. Effect of (CsA + bacterial supernatant) combination on release of IL-1α in HGF. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-1α was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p < 0.05$ compared to control.

4.6. Effect of CsA and bacteria supernatants on IL-6 release:

4.6.1 FIBS cells:

250ng/ml CsA did not cause a significant modulation on release of IL-6 compared to the control (250ng/ml CsA; 314 ± 157 vs control; 264 ± 66pg/ml; $p>0.05$). However, 2000ng/ml CsA significantly increased IL-6 release (2000ng/ml CsA; 392 ± 116pg/ml; Fig 4.11; $p<0.05$ vs control).

P. gingivalis

P.g completely inhibited the release of IL-6 (10.6 ± 0.3pg/ml). However, addition of CsA at either 250ng/ml or 2000ng/ml had no significant effect ($p>0.05$ vs P.g).

A. actinomycetemcomitans

A.a significantly increased the release of IL-6 compared to the controls (A.a; 550 ± 140pg/ml; $p<0.05$ vs control). Furthermore, addition of either 250ng/ml or 2000ng/ml CsA had no significant effect ($p>0.05$).

P. intermedia

P.i had no significant effect on the release of IL-6 compared to the controls (P.i; 434 ± 205pg/ml; $p>0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Addition of 250ng/ml did not significantly modulate the release of IL-6 ($p>0.05$ vs P.i). However, addition of 2000ng/ml CsA significantly increased the IL-6 release compared to that of controls (P.i + 2000ng/ml CsA; 856 ± 255pg/ml; $p<0.05$ vs P.i & 2000ng/ml CsA individually).

F. nucleatum

F.n significantly decreased the release of IL-6 compared to the controls (F.n; 83 ± 70pg/ml; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Addition of 250ng/ml CsA had no significant effect. However, addition of 2000ng/ml CsA significantly increased the release of IL-6 compared to the controls (F.n + 2000ng/ml CsA; 580 ± 177pg/ml; $p<0.05$ vs F.n & 2000ng/ml CsA individually).

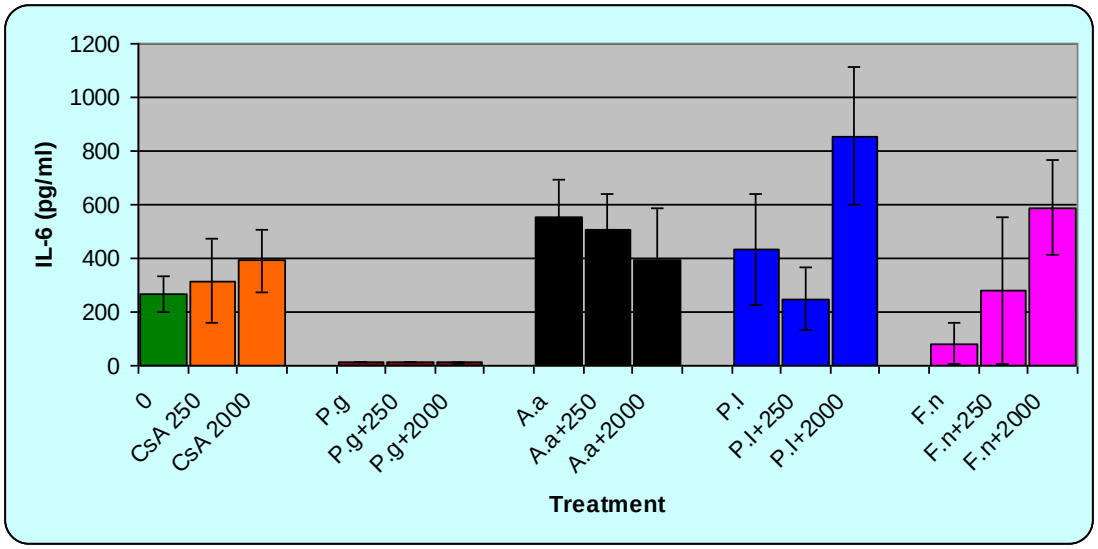


Figure 4.11. Effect of (CsA + bacterial supernatant) combination on release of IL-6 in FIBS. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-6 was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p < 0.05$ compared to control.

4.6.2 OKF6 cells:

250ng/ml CsA did not cause a significant modulation on release of IL-6 compared to the control (250ng/ml CsA; 758 ± 76 vs control; 723 ± 56pg/ml; $p>0.05$). However, 2000ng/ml CsA significantly increased IL-6 release (2000ng/ml CsA; 1118 ± 89pg/ml; Fig 4.12; $p<0.05$ vs control).

P. gingivalis

P.g completely inhibited the release of IL-6 (8.3 ± 1pg/ml). However, addition of CsA at either 250ng/ml or 2000ng/ml had no significant effect ($p>0.05$ vs P.g).

A. actinomycetemcomitans

A.a significantly increased the release of IL-6 compared to the controls (A.a; 1226 ± 91pg/ml; $p<0.05$ vs control & 250ng/ml CsA). However, addition of either 250ng/ml or 2000ng/ml CsA had a limited effect on IL-6 release ($p>0.05$ vs A.a).

P. intermedia

P.i significantly increased the release of IL-6 compared to the control (P.i; 851 ± 69pg/ml; $p<0.05$ vs control). Addition of 250ng/ml CsA did not cause a significant

modulation on the release of IL-6. However, addition of 2000ng/ml CsA significantly decreased IL-6 release compared to the controls (P.i + 2000ng/ml CsA; 562 ± 118pg/ml; $p<0.05$ vs P.i & 2000ng/ml CsA individually).

F. nucleatum

F.n had no significant effect on the release of IL-6 compared to that of control. However, addition of either 250ng/ml or 2000ng/ml CsA did not significantly modulate the IL-6 release.

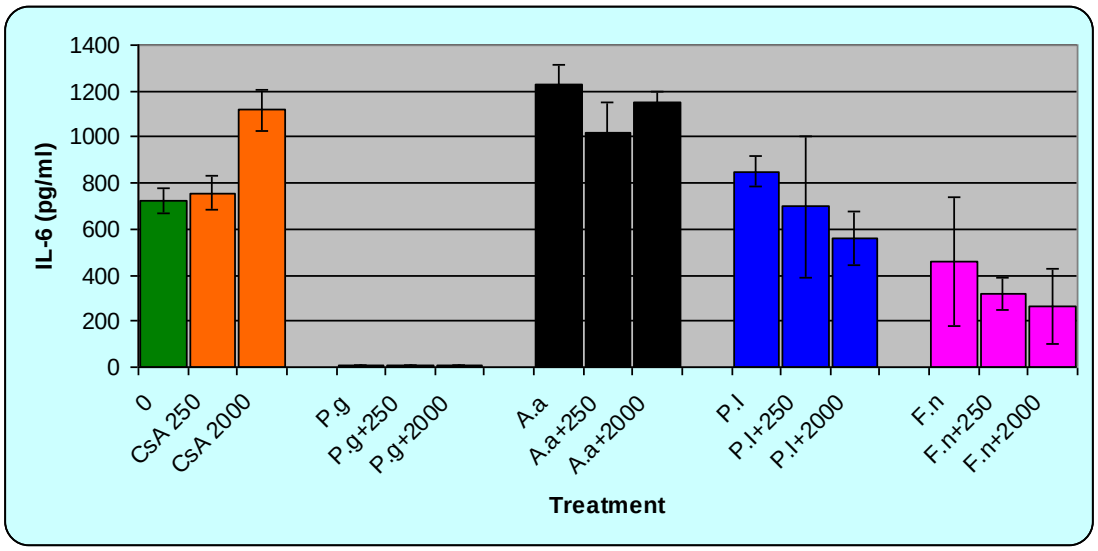


Figure 4.12. Effect of (CsA + bacterial supernatant) combination on release of IL-6 in OKF6. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-6 was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p< 0.05$ compared to control.

4.6.3 TR146 cells:

250ng/ml CsA did not cause a significant modulation on IL-6 release compared to the control (250ng/ml CsA; 110 ± 26 vs control; 89 ± 27pg/ml; $p>0.05$). However, 2000ng/ml CsA significantly increased IL-6 release (2000ng/ml CsA; 152 ± 40pg/ml; Fig 4.13; $p<0.05$ vs control).

P. gingivalis

P.g completely inhibited the release of IL-6 (10 ± 0.5pg/ml). However, addition of CsA at either 250ng/ml or 2000ng/ml had no significant effect ($p<0.05$ vs P.g).

A. actinomycetemcomitans

A.a significantly increased the release of IL-6 compared to the controls (A.a; 711 ± 44pg/ml; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Furthermore, addition of either 250ng/ml or 2000ng/ml CsA did not significantly modulate the release of IL-6 ($p>0.05$ vs A.a).

P. intermedia and F. nucleatum

P.i and F.n supernatants induced a similar effect to that found with A.a supernatant in both individual and CsA-combined exposure.

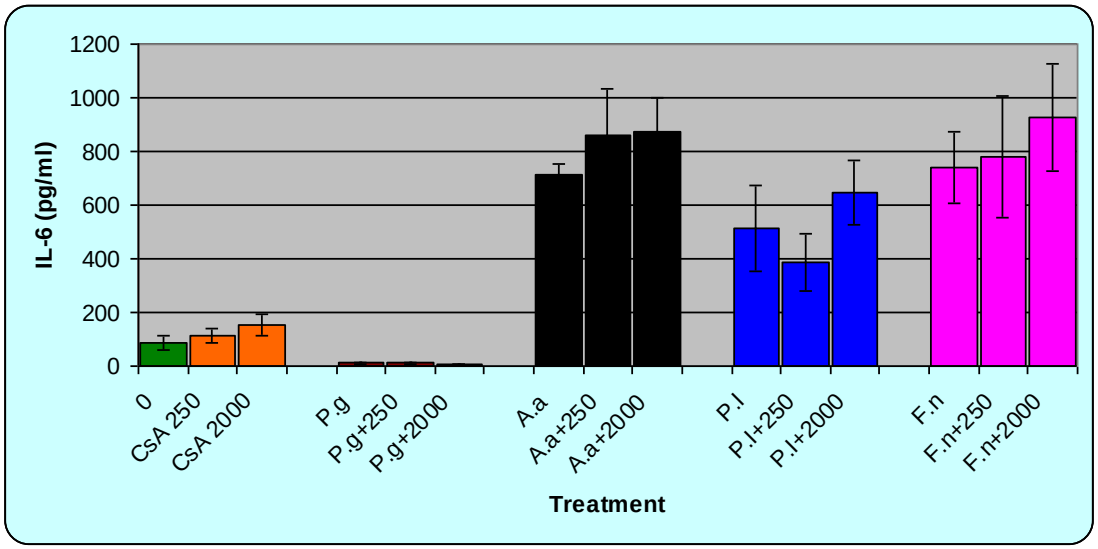


Figure 4.13. Effect of (CsA + bacterial supernatant) combination on release of IL-6 in TR146. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-6 was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p< 0.05$ compared to control.

4.6.4 HGF cells:

250ng/ml CsA did not cause a significant modulation on release of IL-6 compared to the control (250ng/ml CsA; 73 ± 20 vs control; 75 ± 14pg/ml; $p>0.05$). However, 2000ng/ml CsA significantly increased IL-6 release (2000ng/ml CsA; 203 ± 45pg/ml; Fig 4.14; $p<0.05$ vs control).

P. gingivalis

P.g completely inhibited the release of IL-6 (9 ± 0.6pg/ml). However, addition of CsA at either 250ng/ml or 2000ng/ml had no significant effect ($p>0.05$ vs P.g).

A. actinomycetemcomitans

A.a significantly increased the release of IL-6 compared to the controls (A.a; 325 ± 61pg/ml; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Addition of 250ng/ml CsA did not significantly modulate the release of IL-6. However, addition of 2000ng/ml CsA significantly enhanced the stimulatory effect of A.a on IL-6 release (A.a + 2000ng/ml CsA; 522 ± 134pg/ml; $p<0.05$ vs A.a).

P. intermedia

P.i significantly increased the release of IL-6 compared to the control (P.i; 200 ± 38pg/ml; $p<0.05$ vs control & 250ng/ml CsA individually). However, addition of either 250ng/ml or 2000ng/ml CsA significantly enhanced this stimulatory effect with a maximal level of release being found in cultures exposed to a combination of P.i supernatant plus 2000ng/ml CsA (P.i + 250ng/ml CsA; 313 ± 27 & P.i + 2000ng/ml CsA; 545 ± 71pg/ml; $p<0.05$ vs P.i).

F. nucleatum

F.n significantly increased the release of IL-6 compared to the control (F.n; 136 ± 52pg/ml; $p<0.05$ vs control & 250ng/ml CsA individually). Addition of 250ng/ml CsA had no significant effect. However, addition of 2000ng/ml significantly enhanced the stimulatory effect of F.n on IL-6 release (F.n + 2000ng/ml CsA; 388 ± 113pg/ml; $p<0.05$ vs F.n).

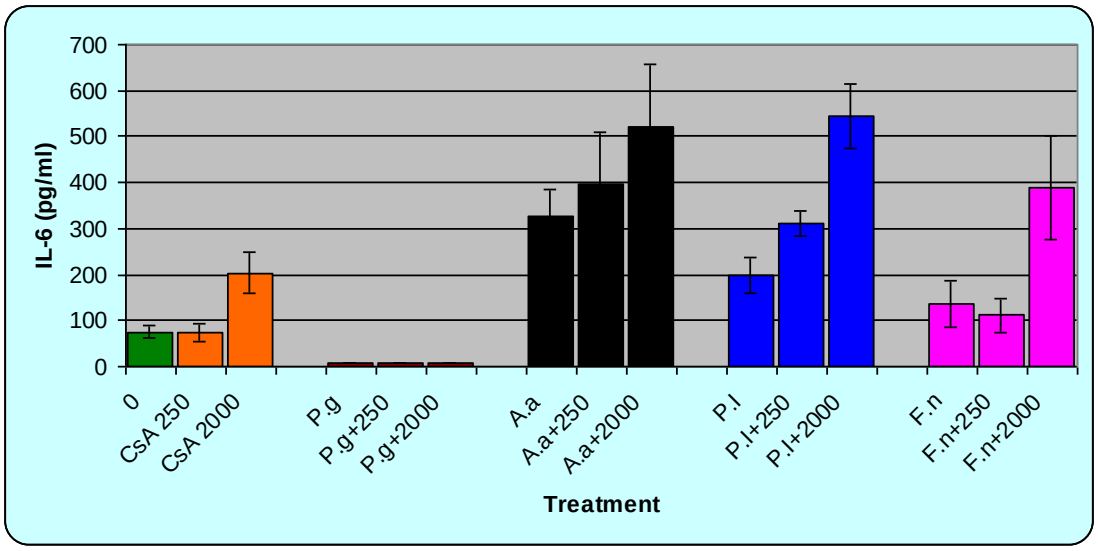


Figure 4.14. Effect of (CsA + bacterial supernatant) combination on release of IL-6 in HGF. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-6 was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p < 0.05$ compared to control.

4.7. Effect of CsA and bacteria supernatants on IL-8 release:

4.7.1 FIBS cells:

250ng/ml or 2000ng/ml CsA had no significant effect on IL-8 release compared to the control (Fig 4.15; $p>0.05$).

P. gingivalis

P.g completely inhibited the release of IL-6 (2 ± 0.7pg/ml). Addition of 250ng/ml CsA had no significant effect ($p>0.05$). However, addition of 2000ng/ml CsA significantly increased the IL-8 release with values still significantly lower than that of control (P.g + 2000ng/ml CsA; 94 ± 49pg/ml; $p<0.05$ vs P.g but $p>0.05$ vs control)

A. actinomycetemcomitans, P. intermedia and F. nucleatum

A.a, P.i, and F.n supernatants had no significant effect on IL-8 release compared to controls. Furthermore, addition of CsA had no significant effect.

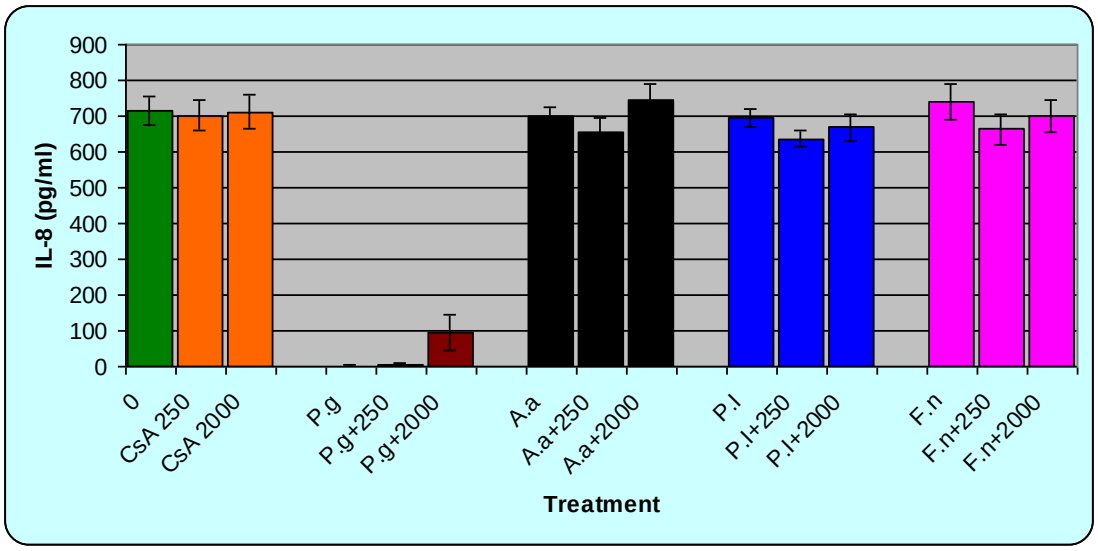


Figure 4.15. Effect of (CsA + bacterial supernatant) combination on release of IL-8 in FIBS. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-8 was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p < 0.05$ compared to control.

4.7.2 OKF6 cells:

250ng/ml or 2000ng/ml CsA did not cause a significant modulation on release of IL-8 compared to the control (785 ± 53 & 856 ± 36 respectively vs control 783 ± 51pg/ml; Fig 4.16; $p>0.05$).

P. gingivalis

P.g completely inhibited the release of IL-8 (3 ± 0.5pg/ml). However, addition of CsA at either 250ng/ml or 2000ng/ml dose had no significant effect (P.g + 250ng/ml;8 ± 5, P.g + 2000ng/ml;4 ± 0.8pg/ml; $p>0.05$ vs P.g).

A. actinomycetemcomitans

A.a significant decreased the release of IL-8 compared to that of controls (722 ± 34pg/ml; $p>0.05$ vs control and both 250ng/ml and 2000ng/ml CsA). Addition of 250ng/ml CsA did not cause a significant modulation on the release of IL-8. However, addition of 2000ng/ml CsA significantly increased IL-8 release with values returning to control level (A.a + 2000ng/ml CsA; 776 ± 44pg/ml).

P. intermedia and F. nucleatum

P.i and F.n had no significant effect on the release of IL-8 compared to that of controls ($p>0.05$). Addition of CsA had no significant effect on the release of IL-8.

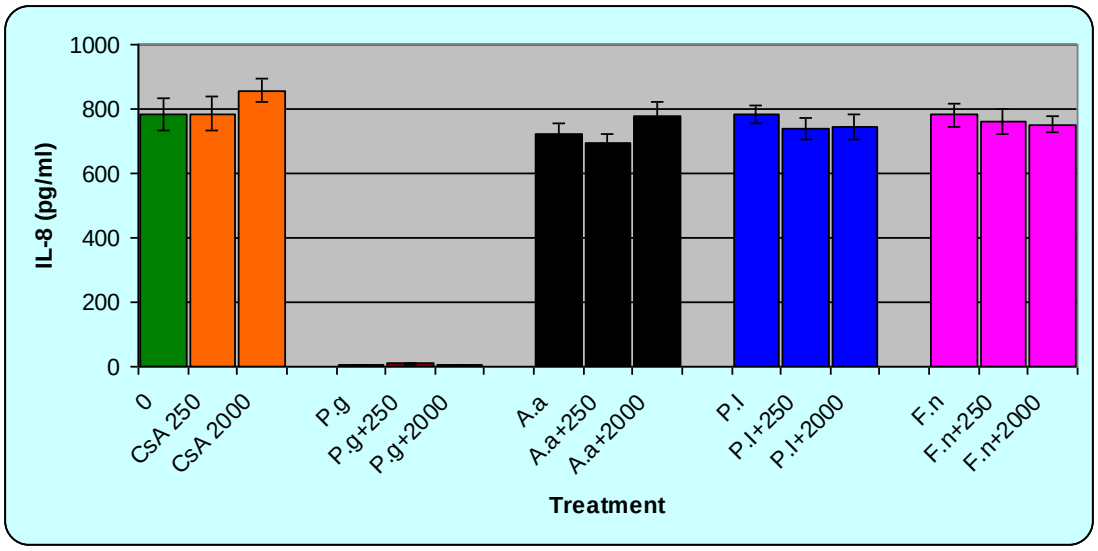


Figure 4.16. Effect of (CsA + bacterial supernatant) combination on release of IL-8 in OKF6. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-8 was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p< 0.05$ compared to control.

4.7.3 TR146 cells:

250ng/ml CsA did not cause a significant modulation on release of IL-8 compared to the control (69 ± 12 vs control; 65 ± 15pg/ml; $p>0.05$). However, 2000ng/ml CsA significantly increased the release of IL-8 compared to the control (82 ± 8pg/ml; Fig 4.17; $p<0.05$).

P. gingivalis

P.g completely inhibited the release of IL-8 (0.3 ± 0.1pg/ml). Addition of CsA at either 250ng/ml or 2000ng/ml did not alter this effect ($p>0.05$ vs P.g).

A. actinomycetemcomitans

A.a significantly increased the release of IL-8 compared to that of controls (104 ± 6pg/ml; $p<0.05$ vs control and both 250ng/ml & 2000ng/ml CsA). Addition of 250ng/ml CsA did not significantly modulate IL-8 release. However, addition of

2000ng/ml CsA significantly enhanced the stimulatory effect of A.a on IL-8 release (A.a + 2000ng/ml CsA; 118 ± 8pg/ml; $p<0.05$ vs A.a & 2000ng/ml CsA individually).

P. intermedia and F. nucleatum

P.i and F.n supernatants significantly increased the release of IL-8 compared to that of controls (P.i; 98 ± 10pg/ml, F.n; 105 ± 8pg/ml; $p<0.05$ vs control). Addition of CsA did not significantly modulate IL-8 release.

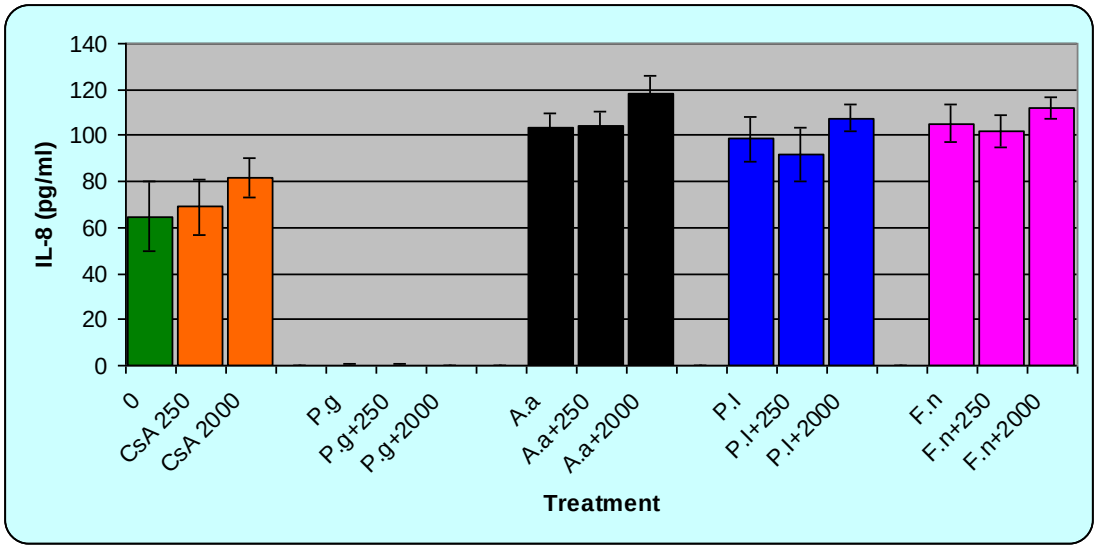


Figure 4.17 Effect of (CsA + bacterial supernatant) combination on release of IL-8 in TR146. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-8 was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p< 0.05$ compared to control.

4.7.4 HGF cells:

250ng/ml CsA did not cause a significant modulation on release of IL-8 compared to the control (159 ± 45 vs control; 114 ± 31pg/ml; $p>0.05$). However, 2000ng/ml CsA caused a significant increase in IL-8 release compared to the control (450 ± 64pg/ml; Fig 4.18; $p<0.05$).

P. gingivalis

P.g completely inhibited the release of IL-8 (1 ± 0.5pg/ml). However, addition of CsA at either 250ng/ml or 2000ng/ml had no significant effect ($p>0.05$ vs P.g).

A. actinomycetemcomitans

A.a significantly increased the release of IL-8 compared to that of control (512 ± 52pg/ml; $p<0.05$ vs control & 250ng/ml). Addition of 250ng/ml CsA did not significantly modulate IL-8 release. However, addition of 2000ng/ml CsA significantly enhanced the stimulatory effect of A.a on IL-8 release (A.a + 2000ng/ml; 657 ± 58pg/ml; $p<0.05$ vs A.a & 2000ng/ml CsA individually).

P. intermedia

P.i significantly increased the release of IL-8 compared to that of control (284 ± 73pg/ml; $p<0.05$ vs control). Addition of 250ng/ml CsA had no significant effect. However, addition of 2000ng/ml CsA significantly enhanced the stimulatory effect of P.i on IL-8 release (P.i + 2000ng/ml; 605 ± 52pg/ml; $p<0.05$ vs P.i & 2000ng/ml CsA individually).

F. nucleatum

F.n induced a similar effect to that found with P.i supernatant in both individual and CsA-combined exposure.

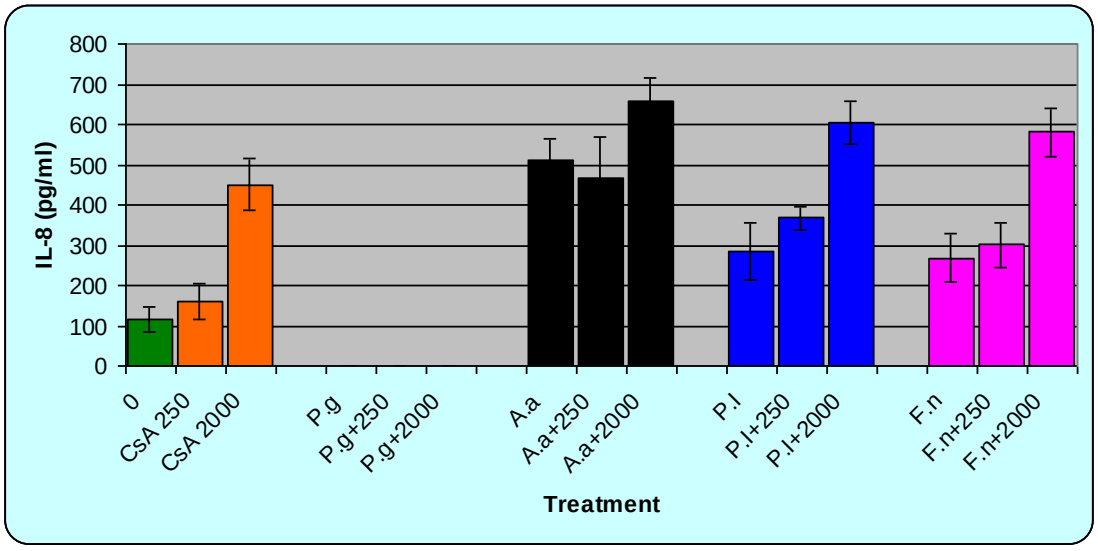


Figure 4.18 Effect of (CsA + bacterial supernatant) combination on release of IL-8 in HGF. Cultures were stimulated with CsA and/or bacterial supernatant. Untreated cells are served as a control. IL-8 was measured by Elisa assay after 24h exposure. Results are expressed as the Mean ± SEM (n=9). Results were considered significant when $p< 0.05$ compared to control.

4.8 Summary:

Effect of CsA and bacterial supernatant combinations on cell viability:

Bacterial supernatants caused different effects on cell viability. An increase in cell viability was found in TR146 (all bacterial supernatants), OKF6 (P.g, F.n, & P.i), and FIBS (A.a, P.i, & F.n). In contrast, P.g caused a significant reduction in FIBS cell viability. Likewise, a significant decrease in cell viability was found in OKF6 treated with A.a. None of the bacterial supernatants had a significant effect on viability of HGF cells. Co-incubation with CsA had no consistent effect on the viability of all cell lines studied.

Effect of CsA and bacterial supernatant combinations on cytokine release:

Bacterial supernatants caused cell type-dependent effects on IL-1α release. A significant increase in IL-1α release was found in TR146 (P.g & P.i) whereas a significant reduction in release of this cytokine was found in FIBS (F.n & P.i) and OKF6 (P.g, P.i, & F.n). A.a, however caused a significant increase in IL-1α release in OKF6. Combinations of 2000ng/ml CsA with bacterial supernatant only caused a significant increase in IL-1α in FIBS and TR146 but not OKF6 and HGF.

P.g supernatant caused a complete inhibition in IL-6 release by all cell lines studied. A.a and P.i however caused a significantly increased release from all the cells. The combination of 2000ng/ml CsA plus supernatant of A.a, P.i, or F.n significantly promoted the release of IL-6 in all cell lines studied. Combinations of 250ng/ml plus bacterial supernatants had a little effect.

P.g supernatant also caused a complete inhibition in IL-8 release in all cell lines studied. A.a, P.i, and F.n significantly increased IL-8 release in TR146 and HGF but had no effect on its release from FIBS and OKF6 cells. However, exposure to combinations of 2000ng/ml plus supernatant of A.a, P.i, or F.n significantly promoted the release of IL-8 in HGF. All combinations had no effect in all other cell lines. Furthermore, combinations of 250ng/ml plus bacterial supernatants had a little effect.

4.9 Conclusion:

The exposure to a combination of bacterial supernatants along with CsA had a little effect on cell viability. Furthermore, the modulation in cytokine release, mostly presented by an increase, was more pronounced in cultures exposed to a combination of bacterial supernatant and 2000ng/ml CsA. There was no clear correlation between the variation in cell viability and the modulation in cytokine release.

The MTT assay however is unable to determine whether the cells are able to replicate and does not differentiate between cycling cells or static cells. Therefore, more sophisticated assays were required to evaluate the cellular response to CsA and bacterial products and whether this response may involve a modulation in the cellular proliferative activity or cell apoptosis or both. Hence, a multi-parameter flowcytometric analysis was developed to analyse: cell cycle, proliferation rate (DNA synthesis), expression of proliferation-related proteins cyclin D1 and cyclin B1, in addition to analyse of expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 after 24h exposure to a combination of bacterial supernatant and CsA (250ng/ml & 2000ng/ml).

Chapter Five

Effect of CsA and bacterial supernatants on cell proliferation and apoptosis in monolayer cell cultures

5.1 Introduction:

This chapter describes the results of the effect of a combination of CsA and bacterial supernatants on cell proliferation and apoptosis assessed using flow cytometry.

5.2 Methods:

The approach used to treat the cells is illustrated in Figure 5.1, whilst the methodology used for the flow cytometry was described in detail in Chapter 2. A brief summary is, however, provided below:

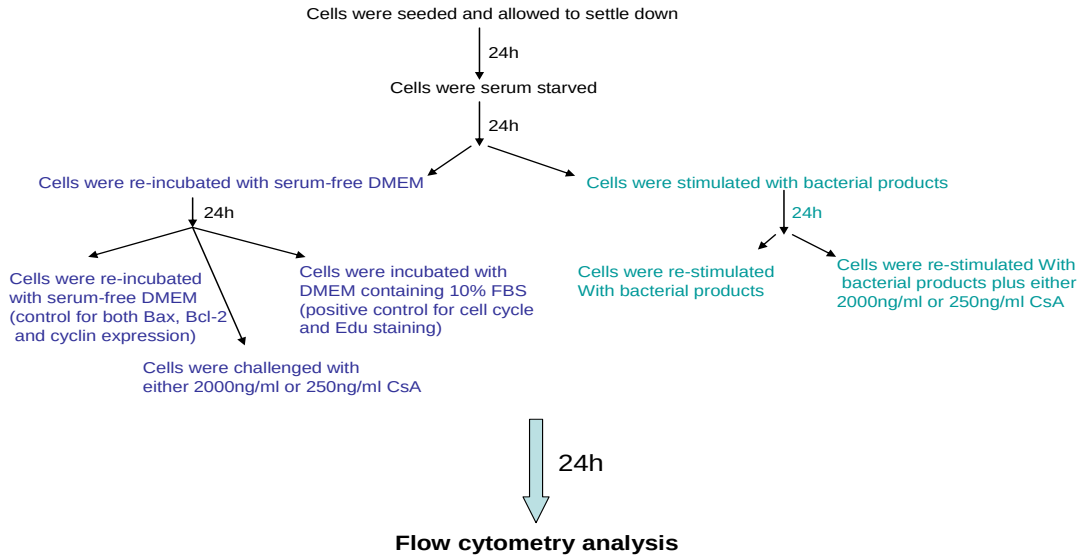


Figure 5.1. Summary of treatment protocol

Cell proliferation was assessed by a four-color flow cytometric analysis, which allowed the simultaneous detection of:

- The proportion of cells in G0/G1, S, or G2/M phase by measuring total DNA content using 7-amino-actinomycin D (7-AAD) (Rabinovitch *et al.,* 1986). The intensity of fluorescence is directly proportional to the amount of DNA within the cell. Distribution into the different phases is thus achieved by

determining the current cellular DNA content; Go/G1 (2C DNA content), S (2C-4C), and G2/M (4C) (Jayat & Ratinaud, 1993).

- Analysis of recently synthesized DNA (S-phase) using click-it Edu system that allows screening the incorporation of Edu (5-ethynyl-2´-deoxyuridine), a nucleoside analogue of thymidine that is incorporated into DNA during active DNA synthesis, using pacific blue fluorescent dye in a click reaction between the ethynyl group of the incorporated Edu in the double-stranded DNA and the small fluorescent azide-containing pacific blue fluorescent dye in presence of copper that catalyzes the click reaction (Buck *et al.*, 2008).
- Cyclin D1 and cyclin B1 expression using specific Alexa Fluor (488 and 647 respectively) conjugated monoclonal antibodies. Cyclin D1 and B1 expression was also analyzed against the cellular DNA levels to relate the expression of these proteins to the cell cycle distribution.

Apoptosis was assessed by evaluating the ratio of Bcl-2 to Bax protein expression using two colour flow cytometry. Bcl-2 and Bax were stained with specific monoclonal antibodies conjugated with Alexa Fluor 488 and 647 respectively (see (Section 2.10 Chapter 2).

An example of the FACS analysis results obtained for cell cycle distribution is illustrated in Fig 5.2 and 5.3 and Bax/Bcl2 expression in Fig 5.4. From these the relative proportions of each parameter and the effect of CsA and bacterial supernatant was calculated. A minimum of 10 – 30 $x10^3$ cells were counted and the proportion for each parameter calculated and expressed as a percentage of the total number of cells.

For clarity the results for each parameter for each cell will be described individually. The plots from the FACS analysis are included in Appendix 1.

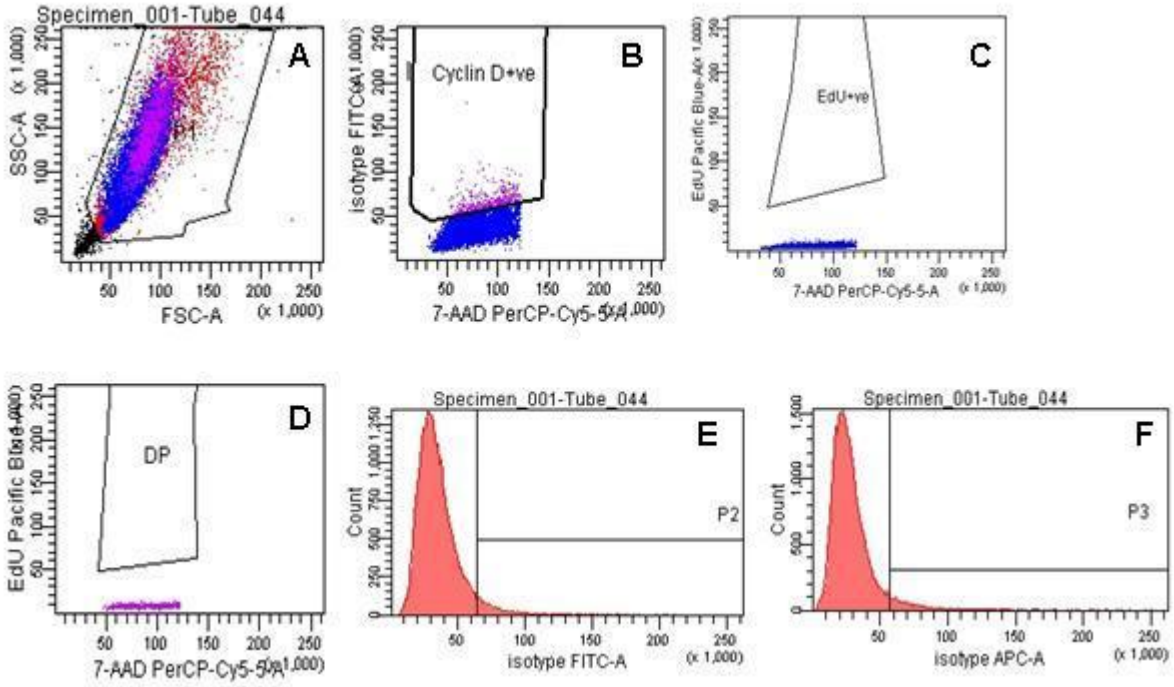


Figure 5.2 Isotype control for evaluating the total DNA content, proliferation rate and detection of the relative levels of cyclin B and cyclin D proteins expression in serum-starved untreated OKF6. (A) Dot plot (FSC-A versus SSC-A) on linear was used to discriminate the single cells and exclude the debris. (B) Isotype matched negative control for cyclin D1 antibody. (C) & (D) negative controls for Edu staining. (E) Single parameter histogram of isotype matched negative control for cyclin D1. (F) Single parameter histogram of isotype matched negative control for cyclin B1.

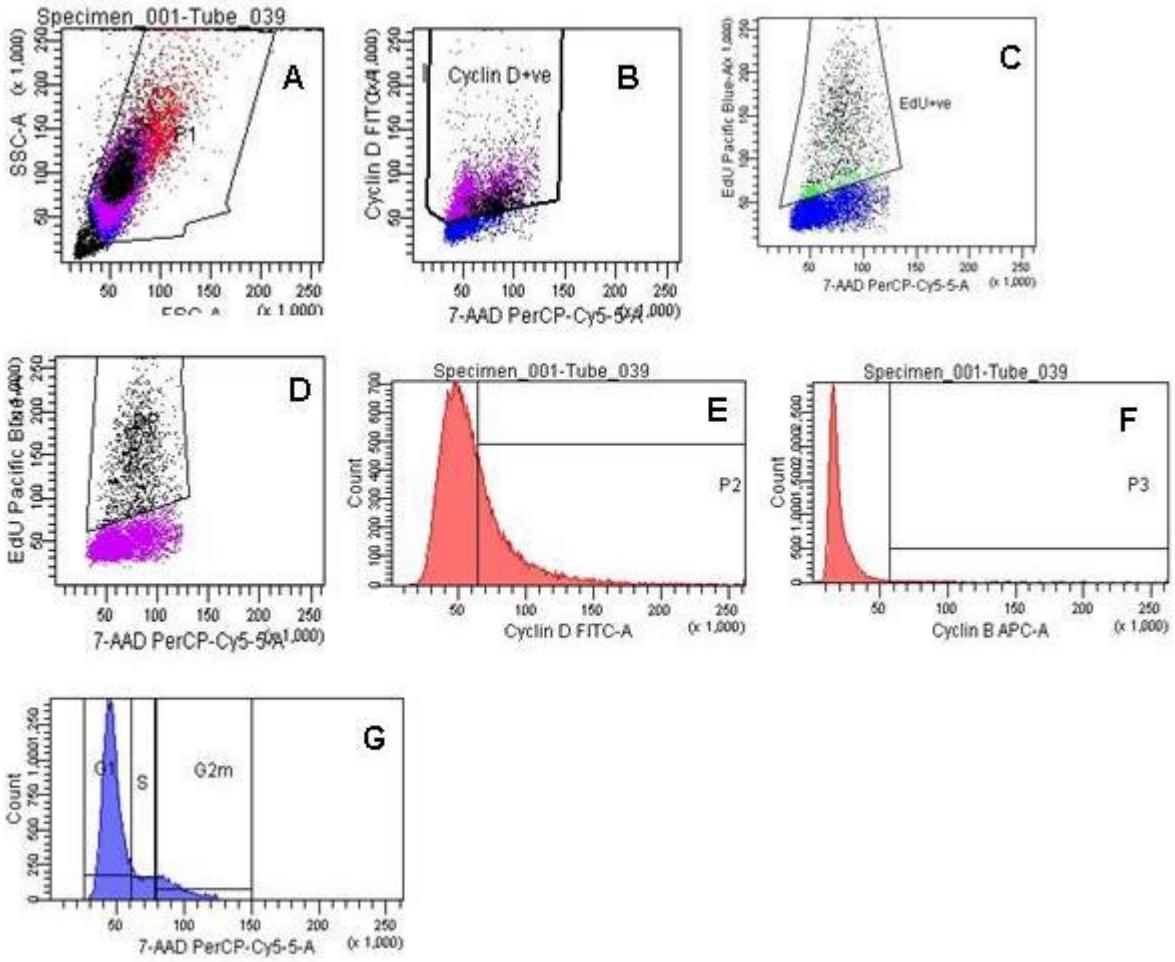


Figure 5.3. Total DNA content, proliferation rate and detection of the relative levels of cyclin B and cyclin D proteins expression in serum-starved untreated OKF6.

Cells were labelled with 10µM Edu for 1hour at 37C. Cells were then processed as described in the materials and methods using anti cyclin D –Alexa fluor 488, anti cyclin B – Alexa fluor 647 and 7-AAD as the cell cycle dye. Cells were analyzed using a BD FACS Canto II. For each histogram/dot plot, the horizontal and vertical markers were set using isotype-matched negative controls. (A) Dot plot (FSC-A versus SSC-A) on linear was used to discriminate the single cells and exclude the debris.(B) Dual parameter dot plot analysing the cyclin D positive cells against DNA content. (C) Dual parameter dot plot analysing the Edu positive cells against DNA content. (D) Dual parameter dot plot analysing the cyclin D positive cells against the Edu positive cells. (E) Single parameter histogram analysing the cyclin D1 positive cells versus cells count. (F) Single parameter histogram analysing the cyclin B1 positive cells versus cells count. (G) Single parameter histogram of DNA content displaying cell cycle distribution.

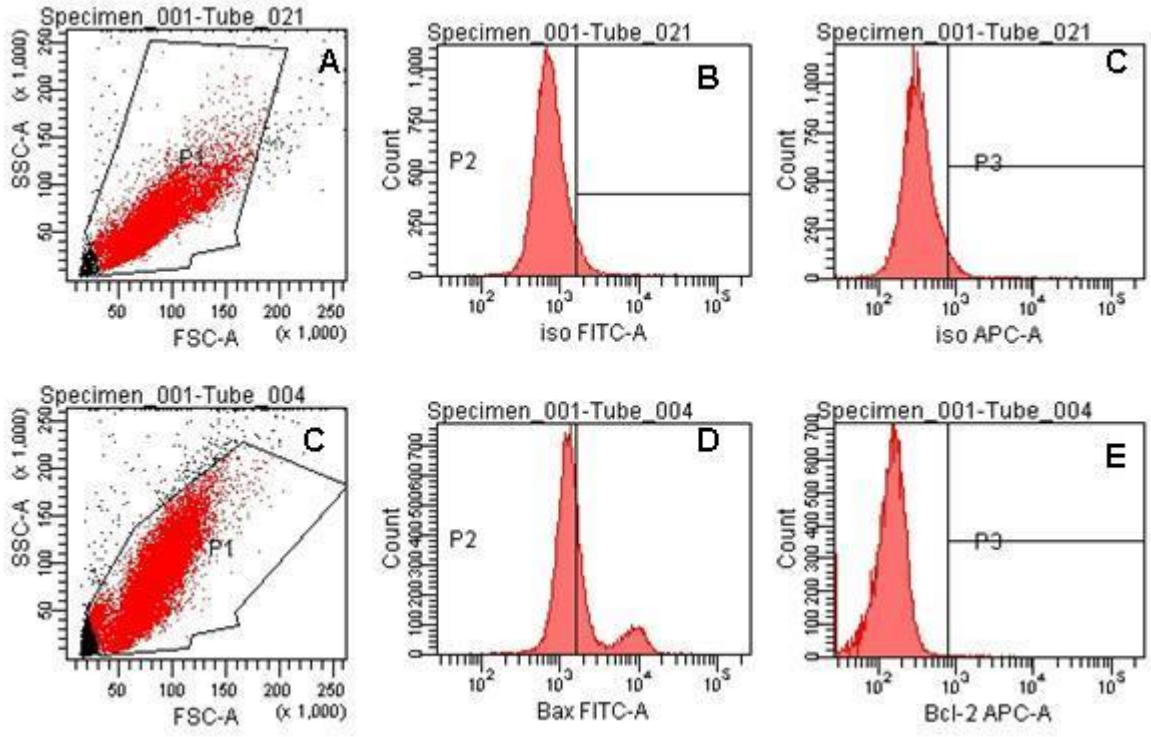


Figure 5.4. Relative levels of Bax and Bcl-2 expression in OKF6 cells after treatment with A.a supernatant for 24h. Isotype matched negative controls for both Bax and Bcl-2 antibodies studied were run firstly to measure the non-specific binding (autofluorescence) and subsequently to determine the position of the positive and negative populations as shown by the vertical markers (upper dot plots). Accordingly, Bax and Bcl-2 expression was measured in OKF6 cells treated with A.a supernatant (lower dot plots).

5.3. FIBS:

5.3.1 Cell cycle distribution:

58% of serum-starved untreated FIBS (Control -) were identified as being in G0/G1 with 16.9%, and 23.3% in S and G2/M respectively (Fig 5.5). Addition of serum to these cells (Control +) increased the proportion of cells in S (20.4%) and G2/M (36.1%) at the expense of G0/G1 (42.1%). CsA (250 ng/ml or 2000ng/ml) had no effect on the proportion of cells in G0/G1, S and G2/M compared to the control (Fig A1, Appendix 2).

P. gingivalis

P.g alone had no effect on the cell cycle distribution. 2000ng/ml CsA, however caused a marked increase in cells in G2/M (34.3%) and a reduction in G0/G1 (44.4%)

A. actinomycetemcomitans

A.a alone or in combination with CsA increased the proportion of cells in G2/M (65%) with a reduction in both S and G0/G1.

P. intermedia

P.i alone or in combination with CsA had no apparent effect on the cell cycle distribution.

F. nucleatum

F.n alone or in combination with CsA had no effect on the cell cycle distribution.

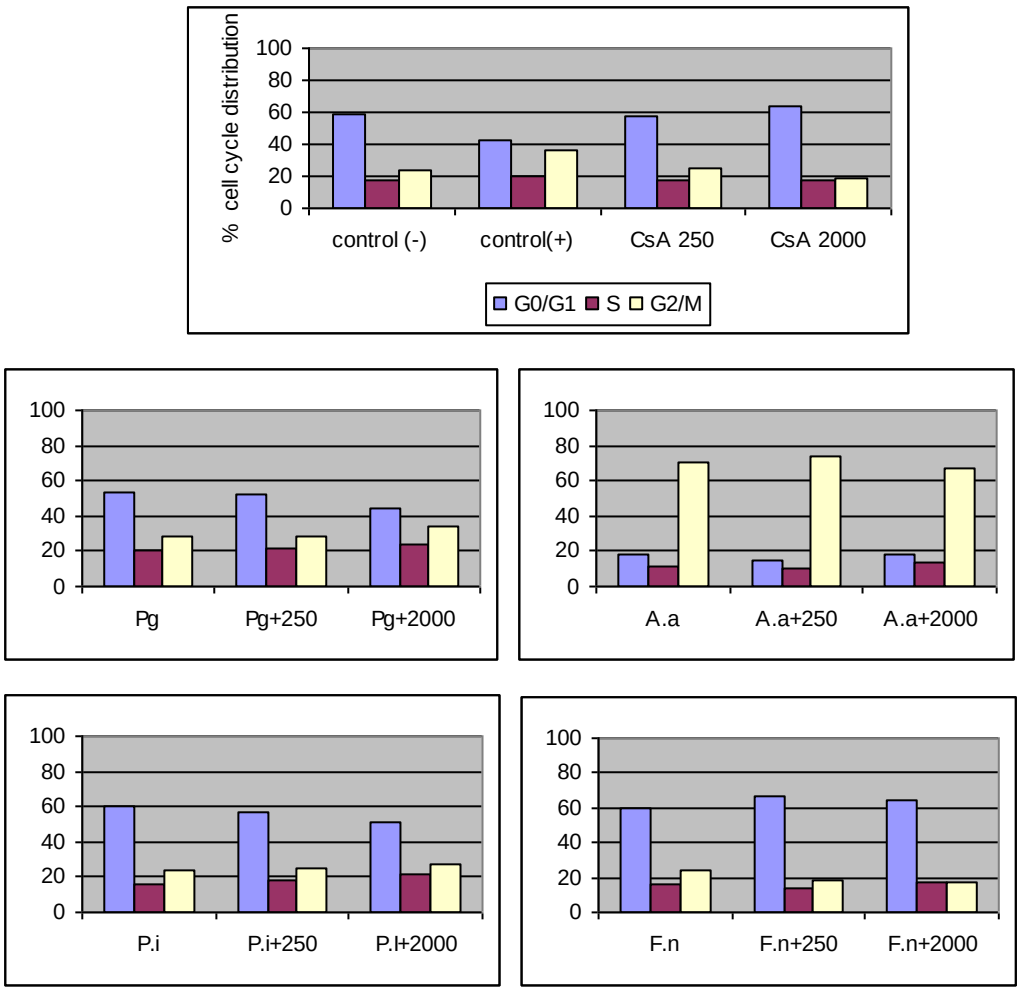


Figure. 5.5. Cell cycle distribution of FIBS cells after treatment with bacterial supernatants and CsA. FIBS cells were stimulated for 24h with CsA and bacterial supernatant and cell cycle distribution assessed by FACS analysis using 7-AAD incorporation. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % cell cycle distribution

5.3.2 Proliferation rate:

The addition of serum to serum free cells caused a 2-fold increase in the proliferation rate (39.9% & 20.8% respectively; Fig 5.6). CsA had no effect on the proliferation rate (Fig A2, Appendix 2).

P. gingivalis

P.g alone or in combination with CsA had no effect on the proliferation rate.

A. actinomycetemcomitans

A.a alone had no effect on the proliferation rate. A.a with 2000ng/ml CsA however increased the proliferation rate of FIBS cells.

P. intermedia

P.i alone had no effect on the proliferation rate. P.i with 2000ng/ml CsA however increased the proliferation rate of FIBS cells.

F. nucleatum

F.n alone or in combination with CsA had no effect on the proliferation rate.

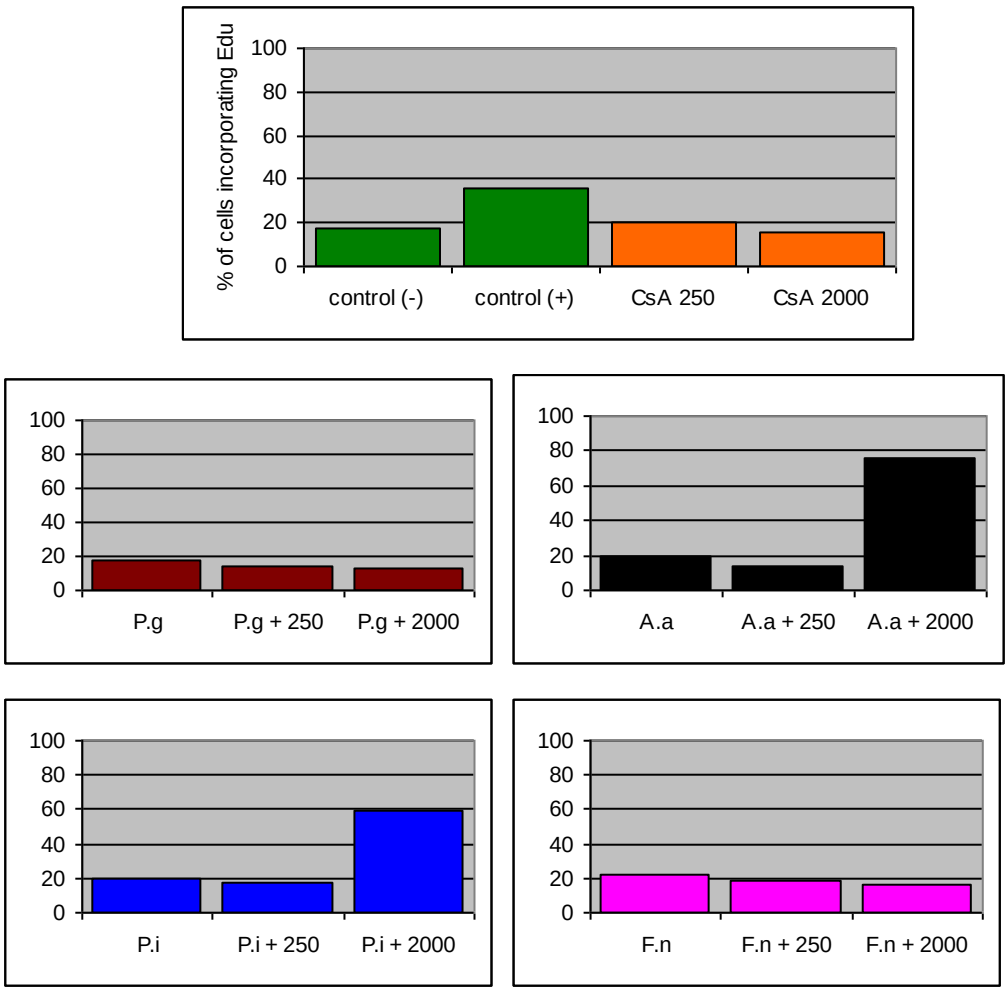


Figure 5.6. Effect of CsA and bacterial supernatants on proliferation rate of FIBS cells. FIBS cells were stimulated for 24h with CsA and bacterial supernatant and proliferation rate assessed by FACS analysis using Edu incorporation. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % of cells incorporating Edu

5.3.3 Cyclin D1 expression:

The addition of serum or CsA (250ng/ml and 2000ng/ml) increased Cyclin D1 expression compared to serum free controls (85%, 45.5%; 53% and 35% respectively; Fig 5.7) & (Fig A3, Appendix 2).

P. gingivalis

P.g alone increased Cyclin D1 expression (80%). This was not affected by the addition of CsA.

A. actinomycetemcomitans

A.a alone increased Cyclin D1 expression (78%). This was not affected by the addition of CsA.

P. intermedia

P.i alone had no effect on Cyclin D1 expression. 2000ng/ml CsA however increased the proportion of Cyclin D1 positive cells (45%).

F. nucleatum

F.n alone reduced Cyclin D1 expression (15%) compared to serum free controls (35%). CsA increased Cyclin D1 expression with 2000ng/ml returning to control levels (40%).

It was revealed from the bivariate dot plot analyzing the cyclin D1 expression versus DNA content that the expression of cyclin D1 in FIBS cells was not exclusive to G1 phase but instead, it was continuous throughout all the cell cycle phases with a maximal level being noticed in cells entering G2/M. This pattern of expression, however, was not affected by any of the treatments applied (Fig 5.8).

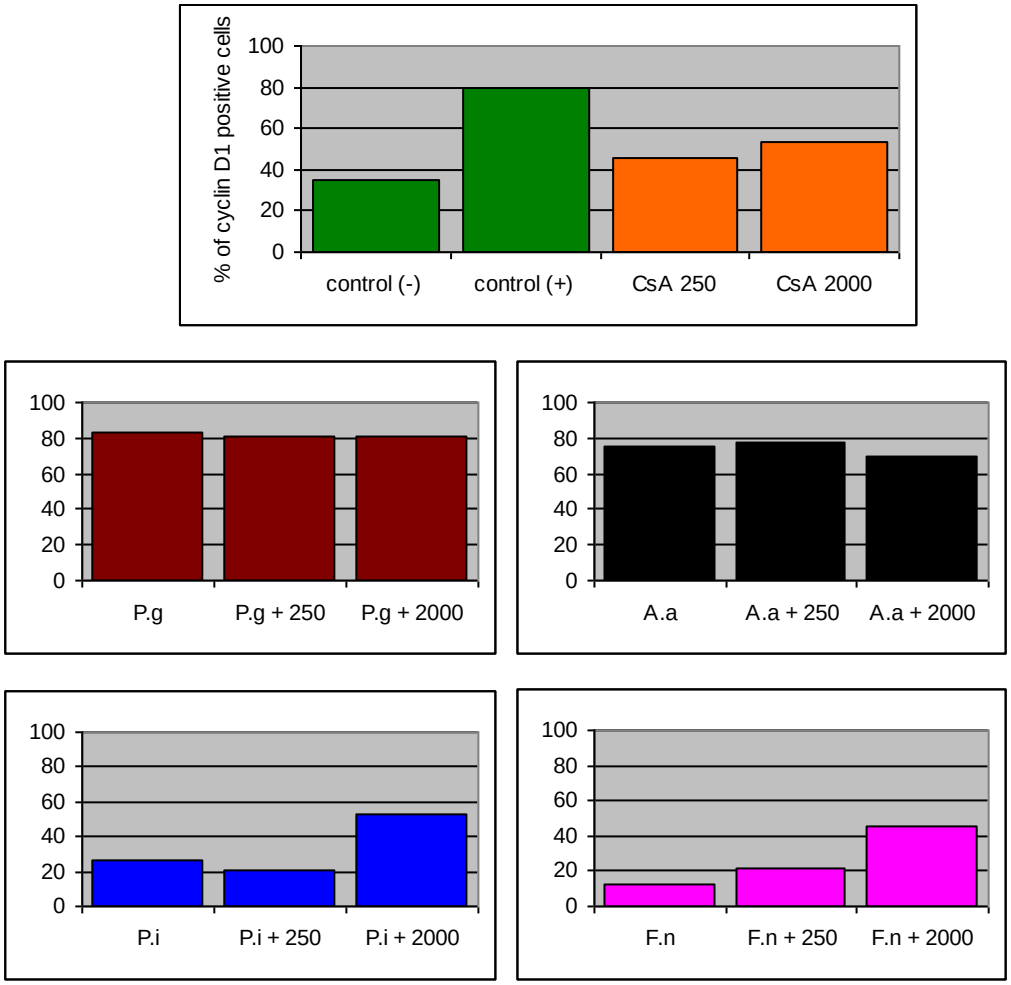


Figure 5.7. Cyclin D1 expression by FIBS exposed to a combination of CsA and bacterial supernatants for 24h. FIBS cells were stimulated for 24h with CsA and bacterial supernatant and Cyclin D1 assessed by FACS analysis. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % of cyclin D1 positive cells

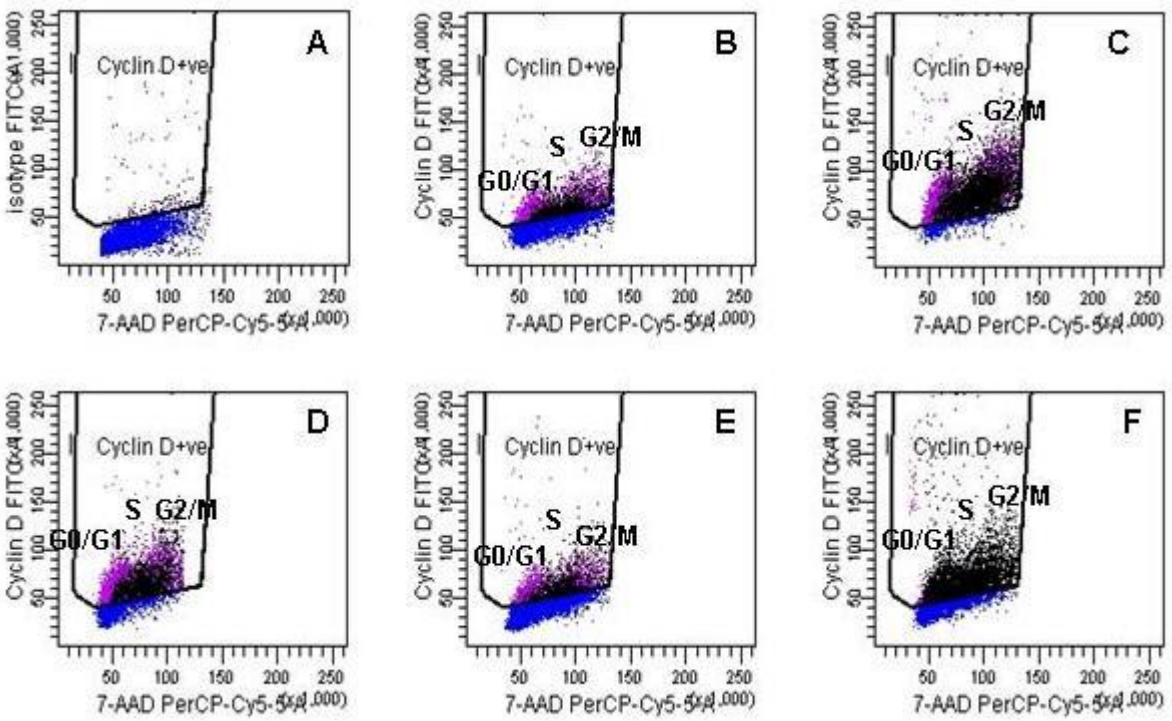


Figure 5.8. Bivariate distributions showing the expression of cyclin D1 in relation to the cell cycle position in FIBS cells after 24h exposure to a combination of CsA plus bacterial supernatants. FIBS cells were stimulated for 24h with CsA and bacterial supernatant and fashion of Cyclin D1 expression was assessed by FACS analysis. Dual parameter dot plots analysing the cyclin D1 positive cells against DNA content were shown. These dot plots show that cyclin D1 was expressed throughout the cell cycle phases. A)- Isotype control was used to determine the positive and negative populations. B)- serum-starved control. C)- serum-rechallenged control. D)- FIBS cells treated with 2000ng/ml CsA. E)- FIBS cells exposed to P.i supernatant. F)- FIBS cells exposed to a combination of P.i supernatant plus 2000ng/ml CsA.

5.3.4 Cyclin B1 expression:

Cyclin B1 was not detected in any of FIBS cultures

5.3.5 Bax expression:

Bax expression was not present in control or CsA treated cells (Fig A10, Appendix 2).

P. gingivalis

P.g alone or in combination with CsA had no effect on Bax expression

A. actinomycetemcomitans

A.a increased Bax expression to 4.5%. This effect was not markedly affected by CsA (Fig 5.9)

P. intermedia

P.i alone or in combination with CsA had no effect on Bax expression

F. nucleatum

F.n alone or in combination with CsA had no effect on Bax expression

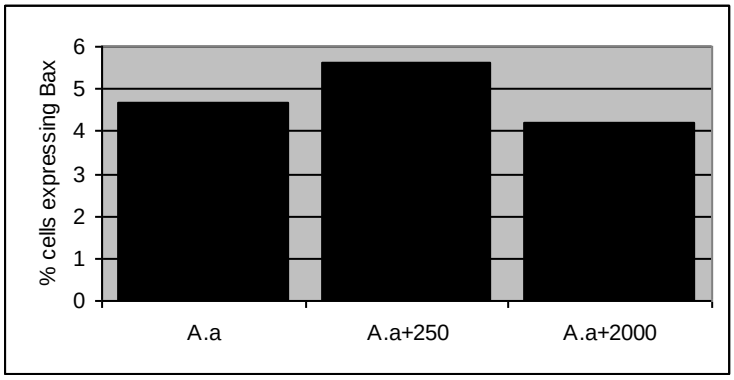


Figure 5.9. Bax expression by FIBS exposed to a combination of CsA and A.a supernatants for 24h. FIBS cells were stimulated for 24h with CsA and bacterial supernatant and Bax expression assessed by FACS analysis. Results are expressed as a proportion of the total number of cells. Bax was not present in control and other supernatants treated cultures.
X axis represents treatment and Y axis represents % cells expressing Bax

5.3.6 Bcl-2 expression:

Bcl-2 was not detected in any of FIBS cultures.

5.4 OKF6:

5.4.1 Cell cycle distribution:

76% of serum starved OKF6 cells were arrested in G0/G1 with 11.2% (S phase), 13.4% (G2/M phase), whilst serum stimulated cells showed a higher proportion in S (34.9%) and G2/M (21.8%) and a lower proportion in G0/G1 (44.3%; Fig 5.10 & Appendix 2). The addition of CsA (250 and 200ng/ml) to the serum free cells did not affect the cell cycle distribution compared to controls.

P. gingivalis

P.g supernatant caused a slight increase in the number of cells in G0/G1 (84.5%) with a parallel decrease in the percentage of S and G2/M fractions. CsA did not affect this distribution.

A. actinomycetemcomitans

A.a products either individually or in combination with CsA markedly increased the percentage of G2/M fraction (64%). This 5 fold higher than that in the serum-starved untreated OKF6 at the expense of G0/G1 fraction (16%).

F. nucleatum and P. intermedia

F.n and P.i supernatants (alone or in combination with CsA) had no effect on the proportion of cells in G0/G1, S or G2/M.

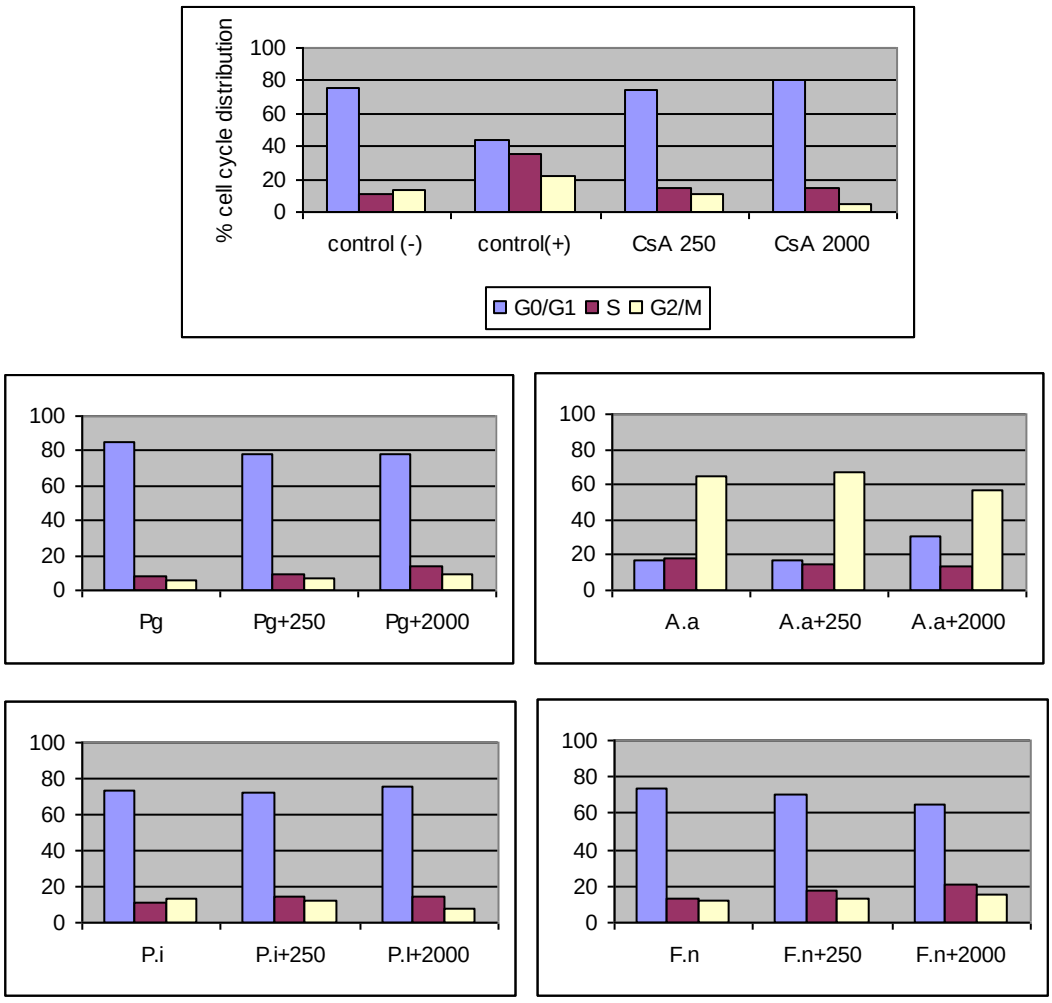


Figure 5.10. Cell cycle distribution of OKF6 cells after treatment with bacterial supernatants and CsA. OKF6 cells were stimulated for 24h with CsA and bacterial supernatant and cell cycle distribution assessed by FACS analysis using 7-AAD incorporation. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % cell cycle distribution

5.4.2 Proliferation rate:

Serum starved cells had a lower level of proliferation (11.4%) compared to those re-challenged with serum (79.9%; Fig 5.11 & Appendix 2). 2000ng/ml CsA increased the proliferation rate to (28.5%), whilst stimulation with 250ng/ml had no effect.

P. gingivalis

P.g supernatant either alone or in combination with 250 ng/ml CsA had no apparent effect on the proliferation rate of OKF6 cells compared to the serum-starved untreated OKF6. Co-stimulated with 2000ng/ml CsA, however decreased the proliferation rate (P.g + 2000ng/ml CsA; 2.7% vs. P.g; 7.9%).

A. actinomycetemcomitans

A.a products caused a 7 fold increase in the proliferation rate compared to that of serum-starved untreated cells (81.8% vs. 11.4%). The addition of CsA to cultures pre-exposed to A.a products decreased the proliferation rate; this was most pronounced with 250ng/ml with the proportion falling to control levels.

P. intermedia

Pi alone or in combination with CsA caused a dose dependent increase in the proliferation rate (39.2%; 59.5 & 89.1% for P.i, 250 and 2000ng/ml CsA respectively).

F. nucleatum

F.n caused a slight increase in the proliferation rate (22%) compared to control (11.4%). 250ng/ml CsA had no effect on the F.n induced change in proliferation, whilst 2000ng/ml CsA reduced the rate to control levels.

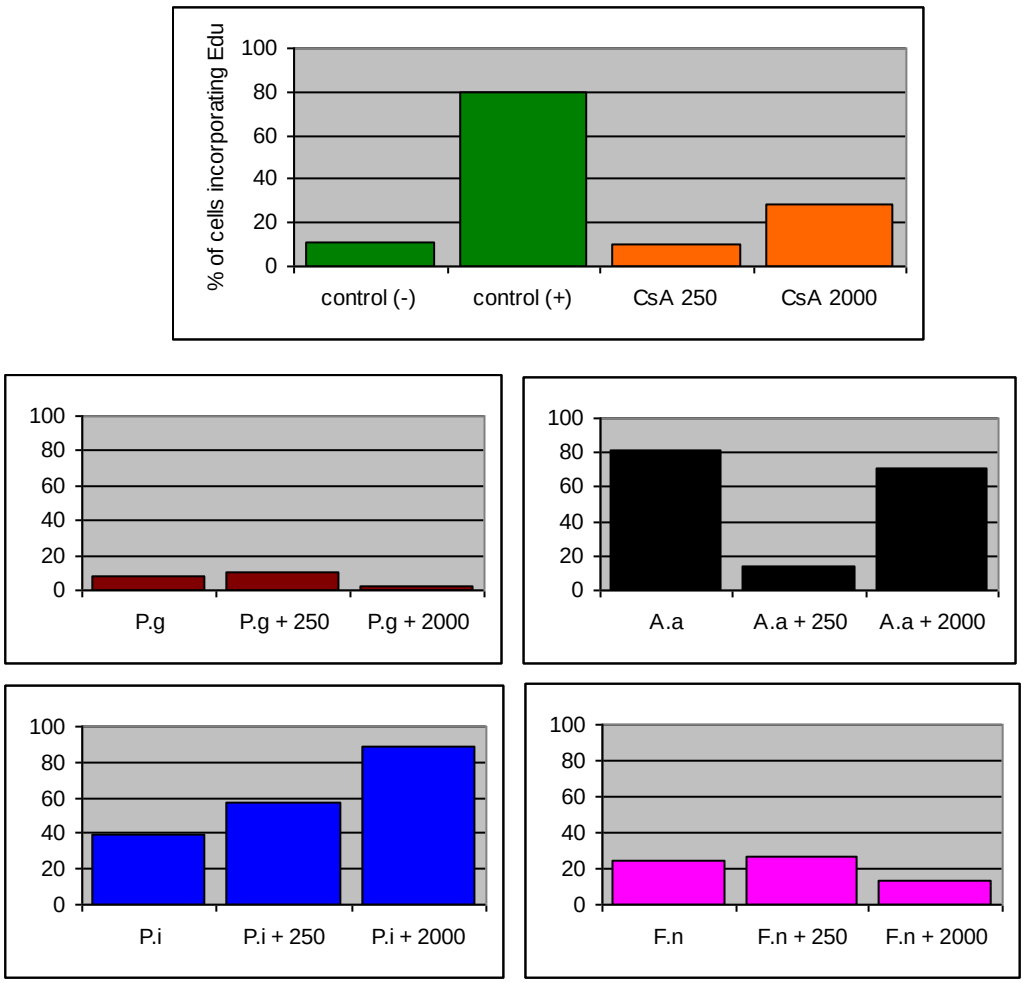


Figure 5.11 Effect of CsA and bacterial supernatants on proliferation rate of OKF6 cells. OKF6 cells were stimulated for 24h with CsA and bacterial supernatant and proliferation rate assessed by FACS analysis using Edu incorporation. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % of cells incorporating Edu

5.4.3 Cyclin D1 expression:

The addition of serum caused a slight increase in Cyclin D1 expression (34.7%) compared to the serum free controls (27.5%), whilst 2000ng/ml CsA caused a marked reduction (13%; Fig 5.12 & Appendix 2).

P. gingivalis

P.g had a limited effect on cyclin D1 expression (Fig 5.12). The addition of 2000ng/ml CsA caused a slight increase in Cyclin D1 expression (40%) compared to P.g alone (24%)

A. actinomycetemcomitans

The maximal levels of cyclin D1 expression were observed in cultures exposed to A.a products either alone (55.4%) or in combination with CsA (250ng/ml – 79.9%; 2000ng/ml CsA; 66.8%).

F. nucleatum

F.n had a limited effect on cyclin D1 expression (Fig 5.12). The addition of 2000ng/ml CsA caused a slight increase in Cyclin D1 expression (36%) compared to F.n. alone (21%).

P. intermedia

P.i, alone or in combination with CsA had no effect on cyclin D1 expression, compared to the untreated starved OKF6.

It was revealed from the bivariate dot plot analyzing the cyclin D1 expression versus DNA content that the expression of cyclin D1 in OKF6 cells was not exclusive to G1 phase but instead, it was continuous throughout all the cell cycle phases. This pattern of expression, however, was not apparently affected by any of the treatments applied (Fig 5.13).

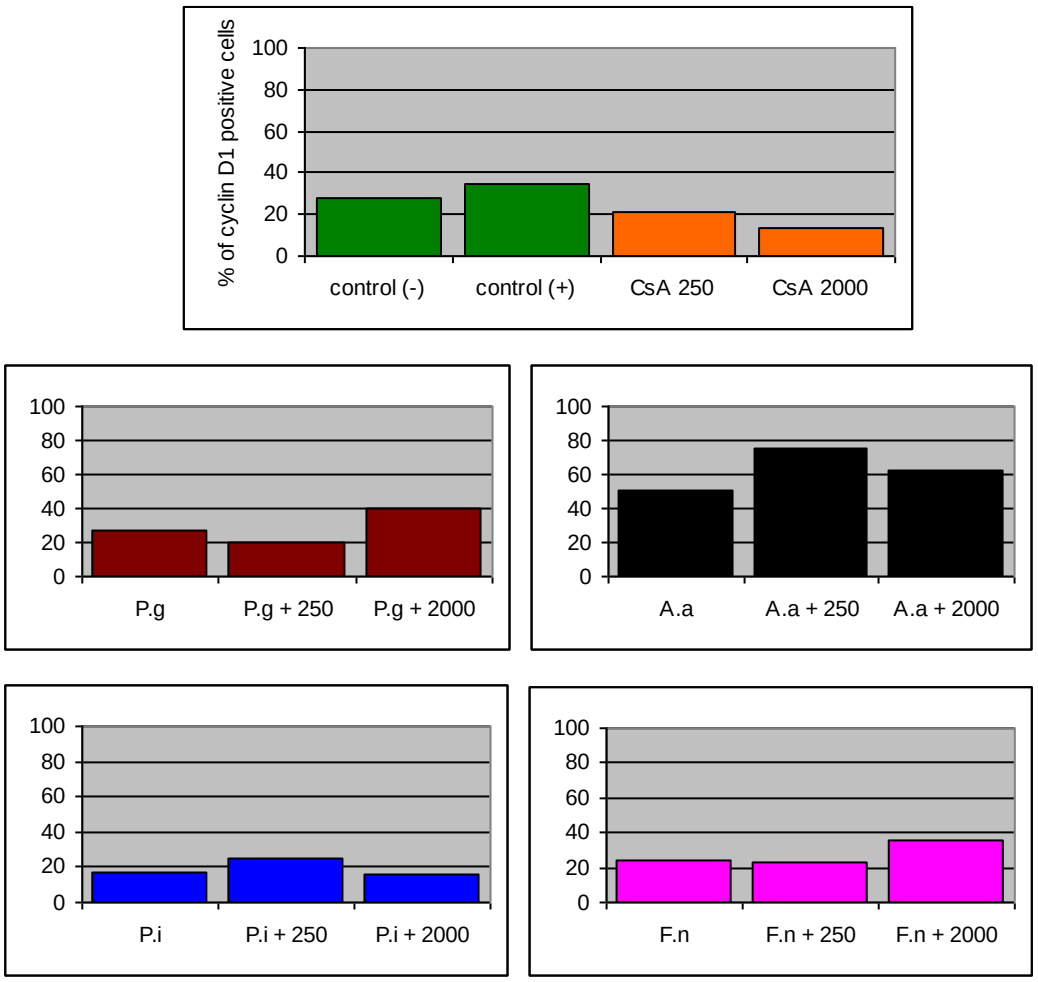


Figure 5.12. Cyclin D1 expression by OKF6 exposed to a combination of CsA and bacterial supernatants for 24h. OKF6 cells were stimulated for 24h with CsA and bacterial supernatants and cyclin D1 assessed by FACS analysis. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % of cyclin D1 positive cells

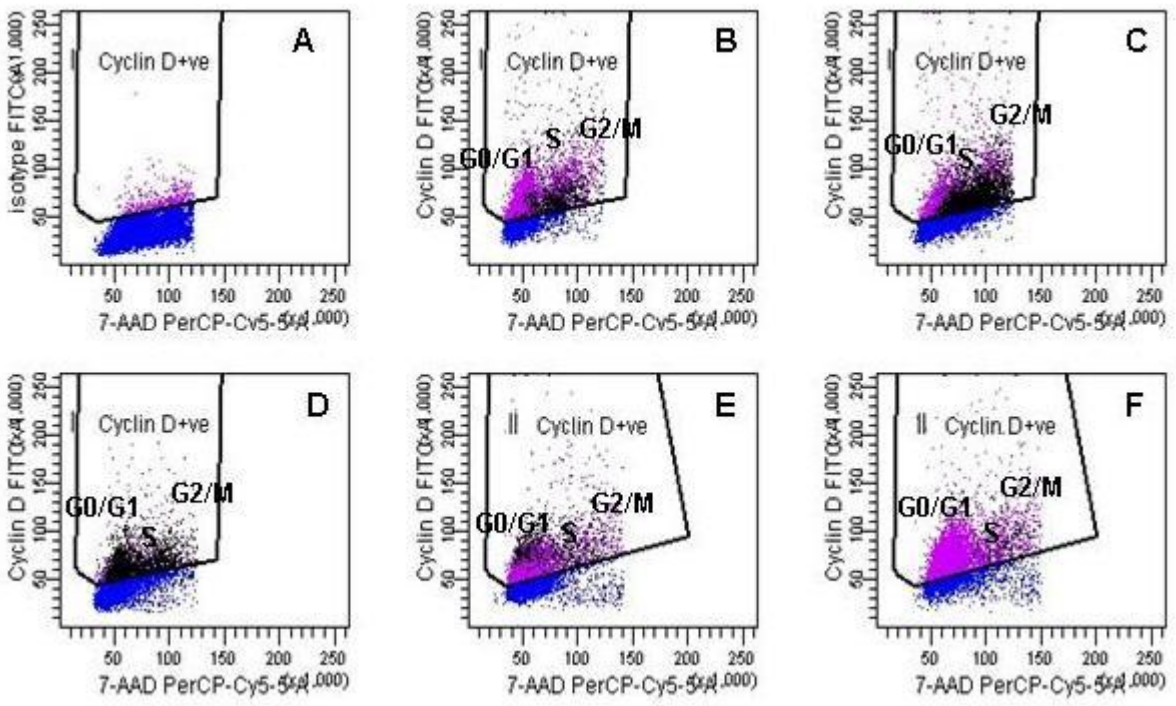


Figure 5.13. Bivariate distributions showing the expression of cyclin D1 in relation to the cell cycle position in OKF6 cells after 24h exposure to a combination of CsA plus bacterial supernatants . OKF6 cells were stimulated for 24h with CsA and bacterial supernatant and fashion of Cyclin D1 expression was assessed by FACS analysis. Dual parameter dot plots analysing the cyclin D1 positive cells against DNA content were shown. These dot plots show that cyclin D1 was expressed throughout the cell cycle phases. A)- Isotype control was used to determine the positive and negative populations. B)- serum-starved control. C)- serum-rechallenged control. D)- OKF6 cells treated with 2000ng/ml CsA. E)- FIBS cells exposed to P.g supernatant. F)- OKF6 cells exposed to a combination of P.g supernatant plus 2000ng/ml CsA.

5.4.4 Cyclin B1 expression:

Cyclin B1 expression was not present in any of the cells studied.

5.4.5 Bax expression:

Low levels of Bax expression was present in control or CsA treated cells (1.6% - 1.8% Fig 5.14) & (Fig A11, Appendix 2).

P. gingivalis

P.g caused a slight increase in Bax expression (5%). CsA caused a slight reduction in this effect (2-3%).

A. actinomycetemcomitans

A.a increased Bax expression (28%). 250ng/ml CsA increased this further to 30% whilst 2000ng/ml CsA reduced the effect (17%).

P. intermedia

P.i alone or in combination with CsA had little or no effect on Bax expression

F. nucleatum

F.n alone or in combination with CsA had little or no effect on Bax expression

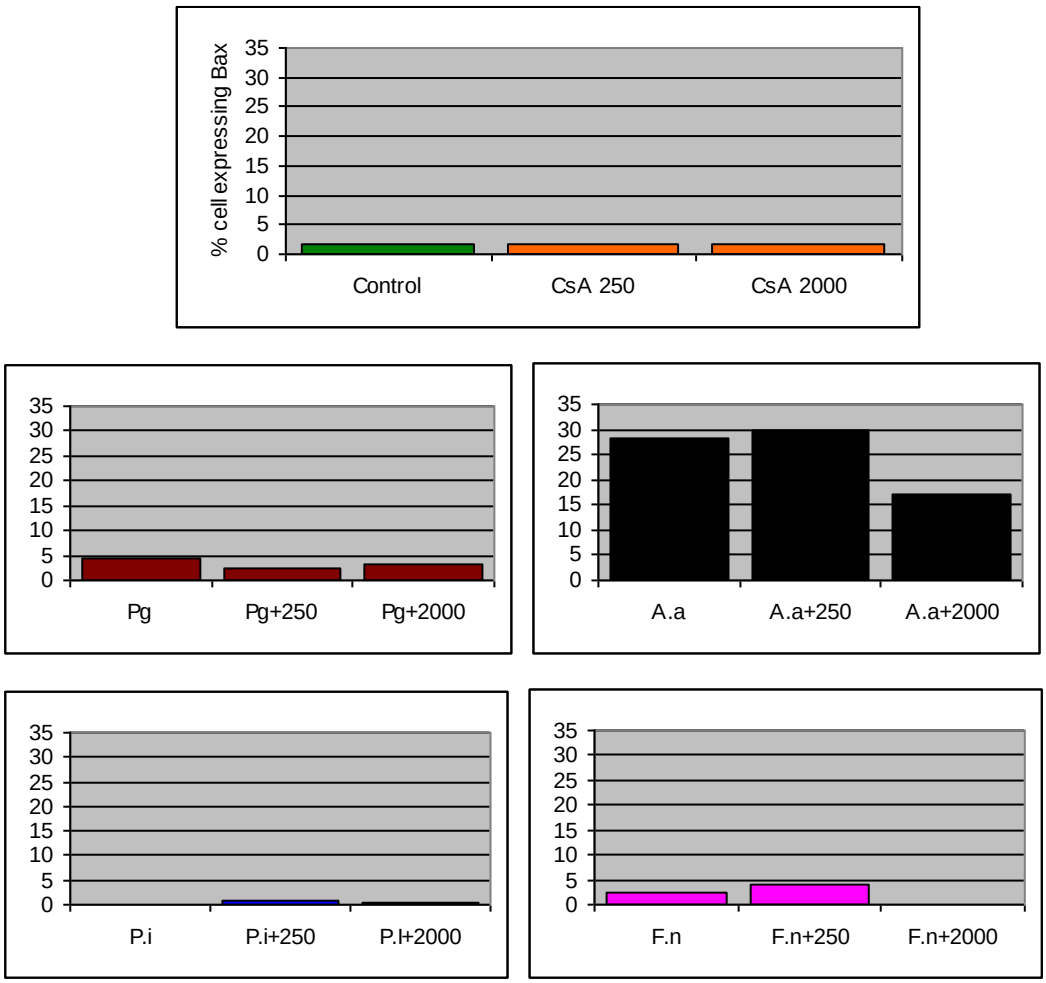


Figure 5.14. Bax expression by OKF6 exposed to a combination of CsA and bacterial supernatants for 24h. OKF6 cells were stimulated for 24h with CsA and bacterial supernatant and Bax assessed by FACS analysis. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % cells expressing Bax

5.4.6 Bcl-2 expression:

Bcl-2 protein was not detected in OKF6 cells (Fig A12, Appendix 2).

5.5. TR146:

5.5.1 Cell cycle distribution:

76.2% of serum starved TR146 were in G0/G1 phase, whilst the addition of serum reduced this proportion to 57.3% and increased S and G2/M (23.3 and 20.2% respectively; Fig 5.15). CsA had a differential effect on the cell cycle: 2000ng/ml CsA caused a marked increase in G0/G1 (82.7%) with a parallel decrease in S and G2/M (11.1% and 6.1% respectively). 250ng/ml CsA increased the proportion in S phase fraction at the expense of G0/G1 (28.2% and 62.3% respectively) & (Fig A4, Appendix 2).

P. gingivalis

P.g alone reduced the proportion of cells in G0/G1 and an increase in S and G2/M compared to serum free controls. The addition of CsA did not alter this effect.

A. actinomycetemcomitans

A.a alone or in combination with CsA increased the proportion of cells in G2/M (40 – 50%) and S (with a reduction in G0/G1).

P. intermedia

P.i had no apparent effect on the cell cycle distribution, although the addition of 2000ng/ml CsA caused a slight increase in G0/G1 fraction (85%)

F. nucleatum

F.n alone reduced G0/G1 and increased S and G2/M. 250ng/ml CsA had no effect on this pattern, whilst 2000ng/ml CsA abrogated the effect.

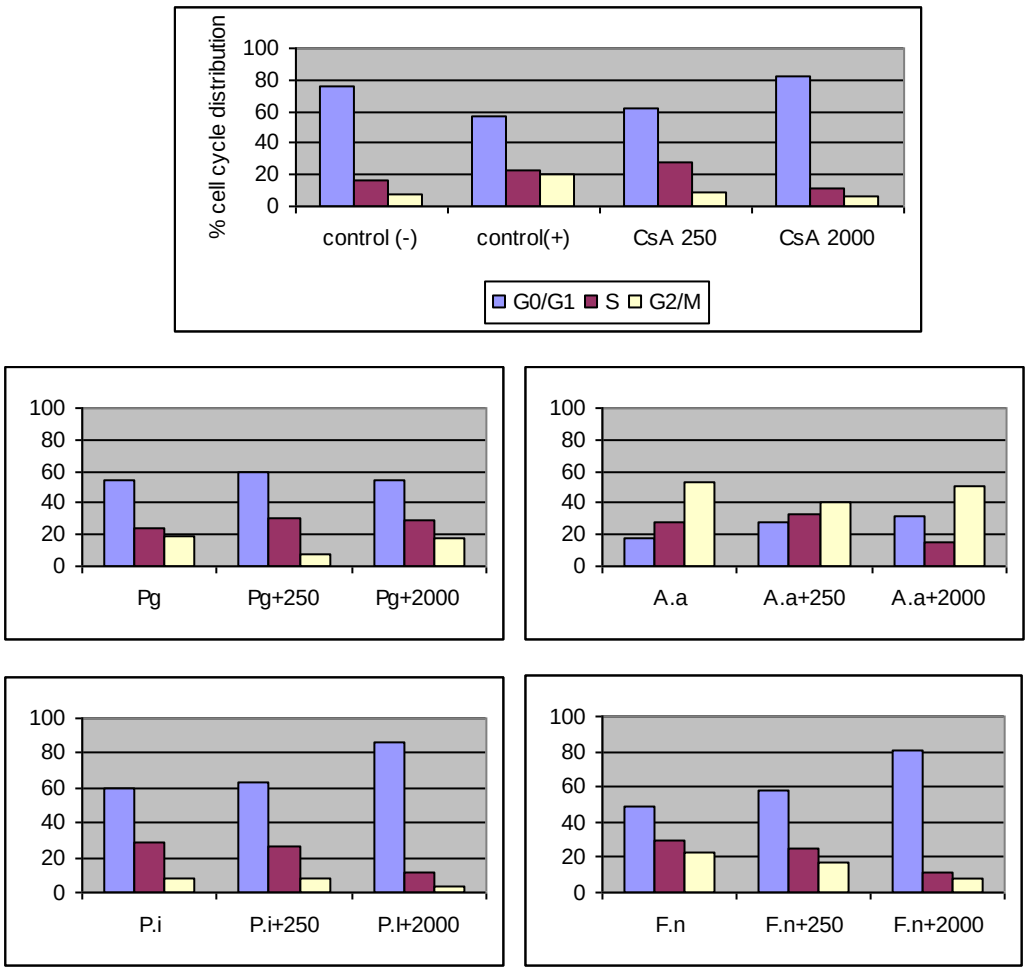


Figure 5.15. Cell cycle distribution of TR146 cells after treatment with bacterial supernatants and CsA. TR146 cells were stimulated for 24h with CsA and bacterial supernatant and cell cycle distribution assessed by FACS analysis using 7-AAD incorporation. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % cell cycle distribution

5.5.2 Proliferation rate:

The addition of serum to serum free cells increased the proliferation rate of TR146 cells (75% & 39.6% respectively; Fig 5.16). 2000g/ml CsA caused a marked reduction (4.5%) whilst 250ng/ml had a similar effect to the addition of serum (Fig A5, Appendix 2).

P. gingivalis

P.g alone or in combination with 250ng/ml CsA had no effect on the proliferation rate. 2000ng/ml however reduced the rate.

A. actinomycetemcomitans

A.a and 250ng/ml CsA increased the proliferation rate, whilst 2000ng/ml CsA however reduced the rate of proliferation.

P. intermedia

P.i alone or in combination with 250ng/ml CsA had no effect on the proliferation rate. 2000ng/ml however reduced the rate.

F. nucleatum

F.n alone or in combination with 250ng/ml CsA had no effect on the proliferation rate. 2000ng/ml however reduced the rate.

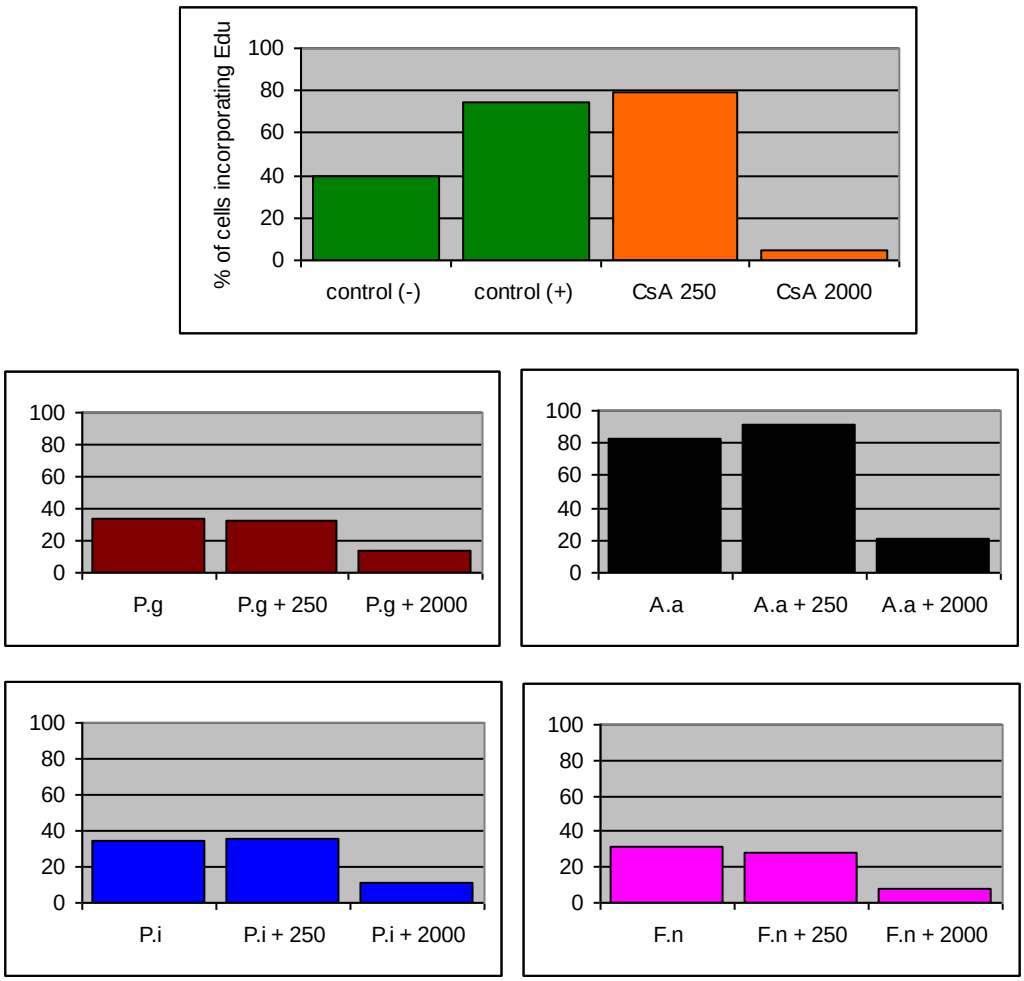


Figure 5.16. **Effect of CsA and bacterial supernatants on proliferation rate of TR146 cells.** TR146 cells were stimulated for 24h with CsA and bacterial supernatant and proliferation rate assessed by FACS analysis using Edu incorporation. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % of cells incorporating Edu

5.5.3 Cyclin D1 expression:

The addition of serum increased Cyclin D1 expression (58%) compared to serum free controls (49% respectively, Fig 5.17). CsA had no effect on Cyclin D (Fig A6, Appendix 2).

P. gingivalis

P.g reduced Cyclin D expression (35%). This was not affected by the addition of CsA.

A. actinomycetemcomitans

A.a increased Cyclin D1 expression (60%). This was not affected by the addition of CsA.

P. intermedia

P.i alone or in combination with CsA had no effect on Cyclin D1 expression.

F. nucleatum

F.n alone or in combination with CsA had no effect on Cyclin D1 expression.

It was revealed from the bivariate dot plot analyzing the cyclin D1 expression versus DNA content that the expression of cyclin D1 in TR146 cells was expressed mainly in G0/G1 and cells entering S phase. This pattern of expression, however, was not affected by any of the treatments applied (Fig 5.18).

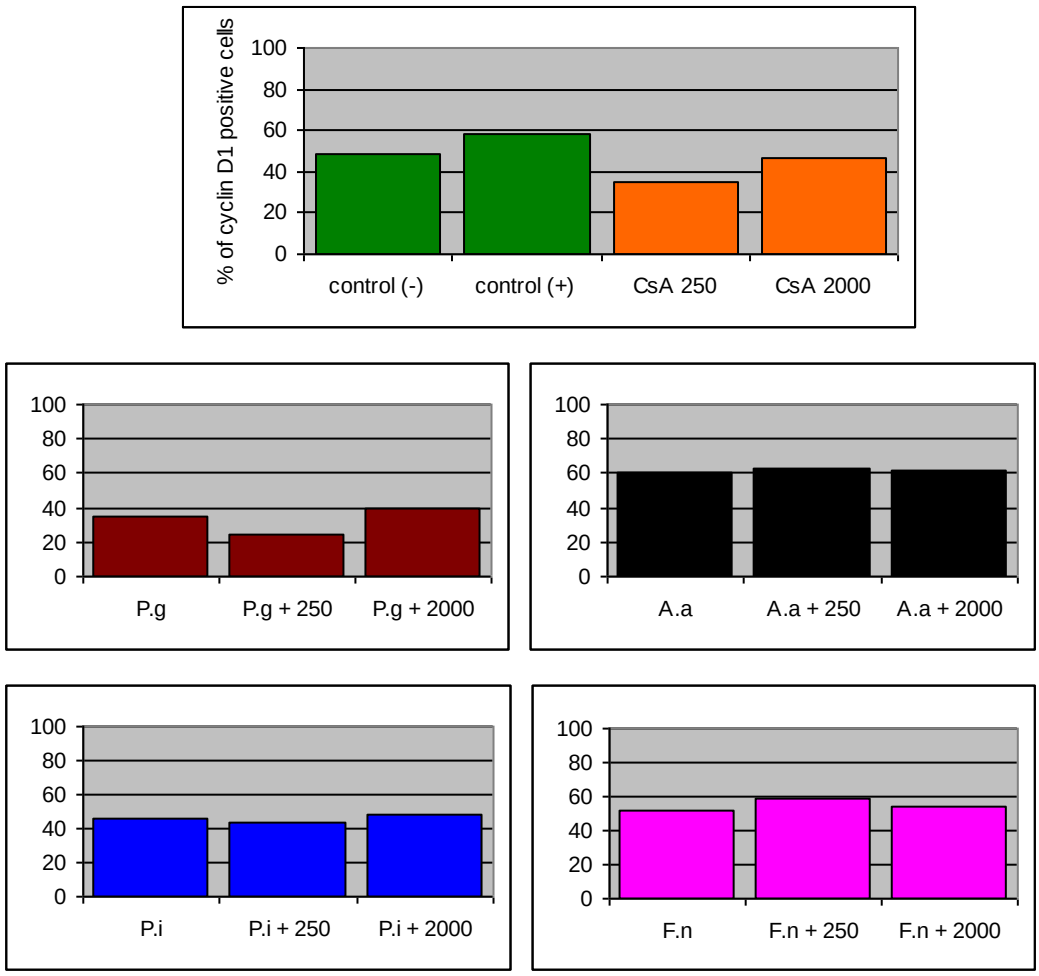


Figure 5.17. Cyclin D1 expression by TR146 exposed to a combination of CsA and bacterial supernatants for 24h. TR146 cells were stimulated for 24h with CsA and bacterial supernatant and cyclin D1 assessed by FACS analysis. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % of cyclin D1 positive cells

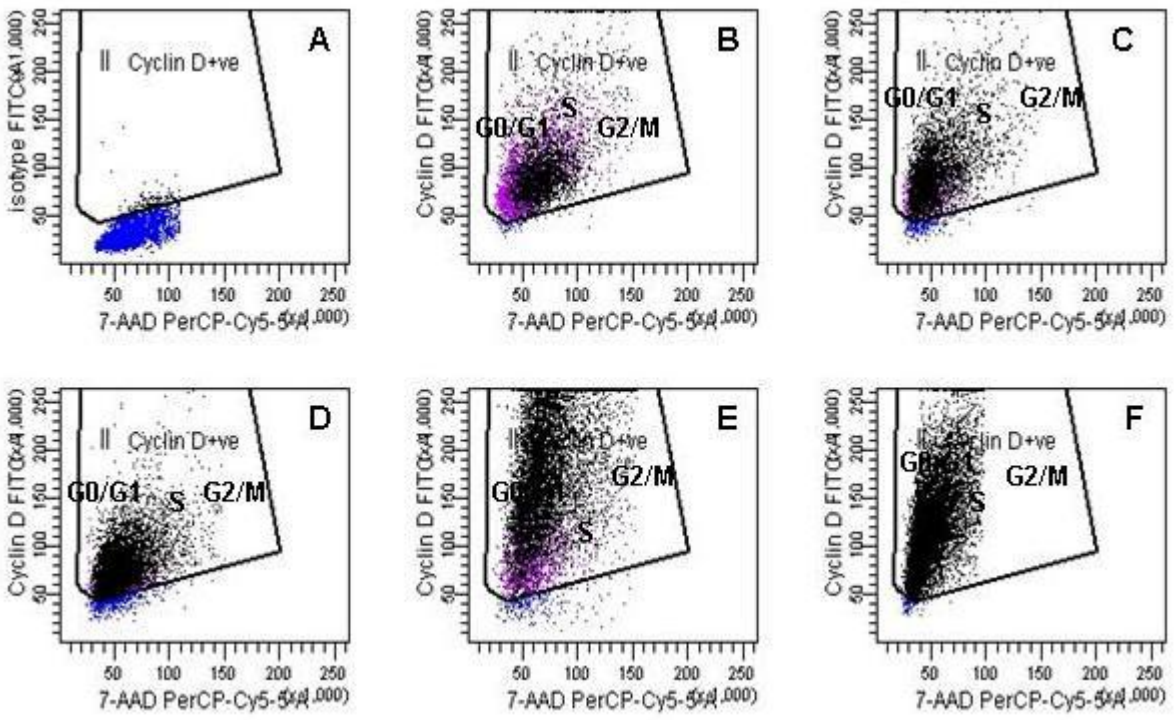


Figure 5.18. Bivariate distributions showing the expression of cyclin D1 in relation to the cell cycle position in TR146 cells after 24h exposure to a combination of CsA plus bacterial supernatants. TR146 cells were stimulated for 24h with CsA and bacterial supernatant and fashion of Cyclin D1 expression was assessed by FACS analysis. Dual parameter dot plots analysing the cyclin D1 positive cells against DNA content were shown. These dot plots show that cyclin D1 was expressed throughout the cell cycle phases. A)- Isotype control was used to determine the positive and negative populations. B)- serum-starved control. C)- serum-rechallenged control. D)- TR146 cells treated with 2000ng/ml CsA. E)- FIBS cells exposed to P.i supernatant. F)- TR146 cells exposed to a combination of P.i supernatant plus 2000ng/ml CsA.

5.5.4 Cyclin B1 expression:

Cyclin B1 was not detected in any of TR146 cultures

5.5.5 Bax expression:

Bax expression was not present in control or CsA treated cells (Fig A13, Appendix 2).

P. gingivalis

P.g alone or in combination with CsA had no effect on Bax expression

A. actinomycetemcomitans

A.a increased Bax expression to 3.9%, whilst 2000ng/ml CsA increased this further to 7.5% (Fig 5.19).

P. intermedia

P.i alone or in combination with CsA had no effect on Bax expression

F. nucleatum

F.n alone or in combination with CsA had no effect on Bax expression

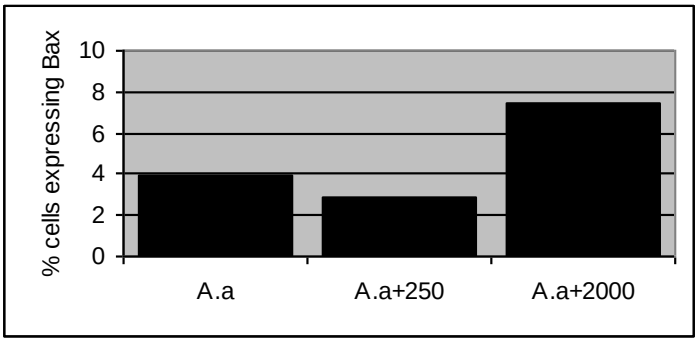


Figure 5.19. **Bax expression by TR146 exposed to a combination of CsA and A.a supernatants for 24h**. TR146 cells were stimulated for 24h with CsA and bacterial supernatant and Bax assessed by FACS analysis. Results are expressed as a proportion of the total number of cells. Bax was not present in control and other supernatants treated cultures.

5.5.6 Bcl-2 expression:

Bcl-2 was not detected in any of TR146 cultures.

5.6 HGF:

5.6.1 Cell cycle distribution:

The addition of serum or 2000ng/ml CsA had little effect on the proportion of cells in G0/G1; S and G2/M (Fig 5.20) & (Fig A7, Appendix 2).

P. gingivalis

P.g alone reduced the proportion of cells in G0/G1 and an increase in S compared to serum free controls. The addition of CsA did not alter this effect.

A. actinomycetemcomitans

A.a alone reduced the proportion of cells in G0/G1 and an increase in S compared to serum free controls. The addition of CsA did not alter this effect.

P. intermedia

P.i alone reduced the proportion of cells in G0/G1 and an increase in S compared to serum free controls. The addition of CsA did not alter this effect.

F. nucleatum

F.n alone reduced the proportion of cells in G0/G1 and an increase in S compared to serum free controls. The addition of CsA did not alter this effect.

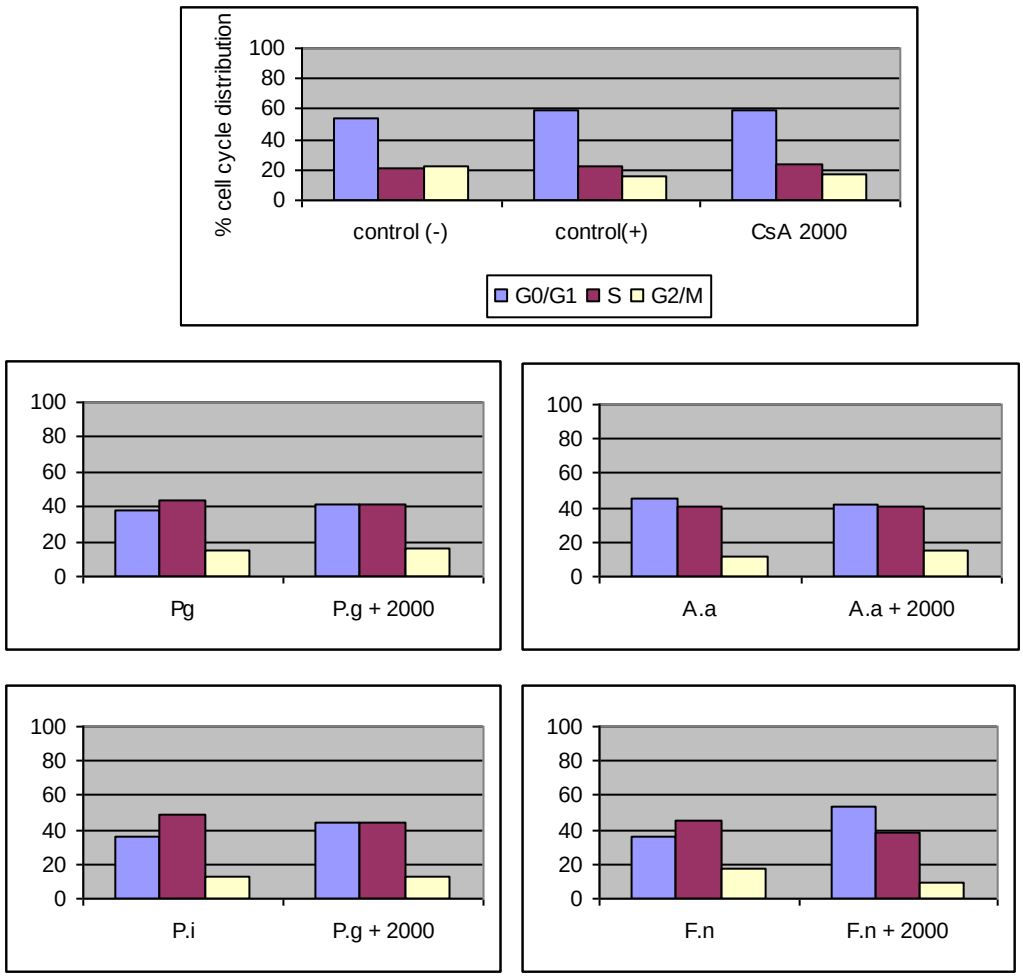


Figure 5.20. Cell cycle distribution of HGF cells after treatment with bacterial supernatants and CsA. HGF cells were stimulated for 24h with CsA and bacterial supernatant and cell cycle distribution assessed by FACS analysis using 7-AAD incorporation. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % cell cycle distribution

5.6.2 Proliferation rate:

The addition of serum or 2000ng/ml to serum free cells increased the proliferation rate of HGF cells (66%, 34% & 20.4% respectively; Fig 5.21) & (Fig A8, Appendix 2).

P. gingivalis

P.g increased the proliferation rate to 42.1%, whilst 2000ng/ml CsA reduced this rate to 34.8%

A. actinomycetemcomitans

A.a increased the proliferation rate to 46%, whilst 2000ng/ml CsA increased this further to 58.6%

P. intermedia

P.i increased the proliferation rate to 38%, whilst 2000ng/ml CsA increased this further to 65%

F. nucleatum

F.n increased the proliferation rate to 52%, whilst 2000ng/ml CsA reduced this effect to 36%

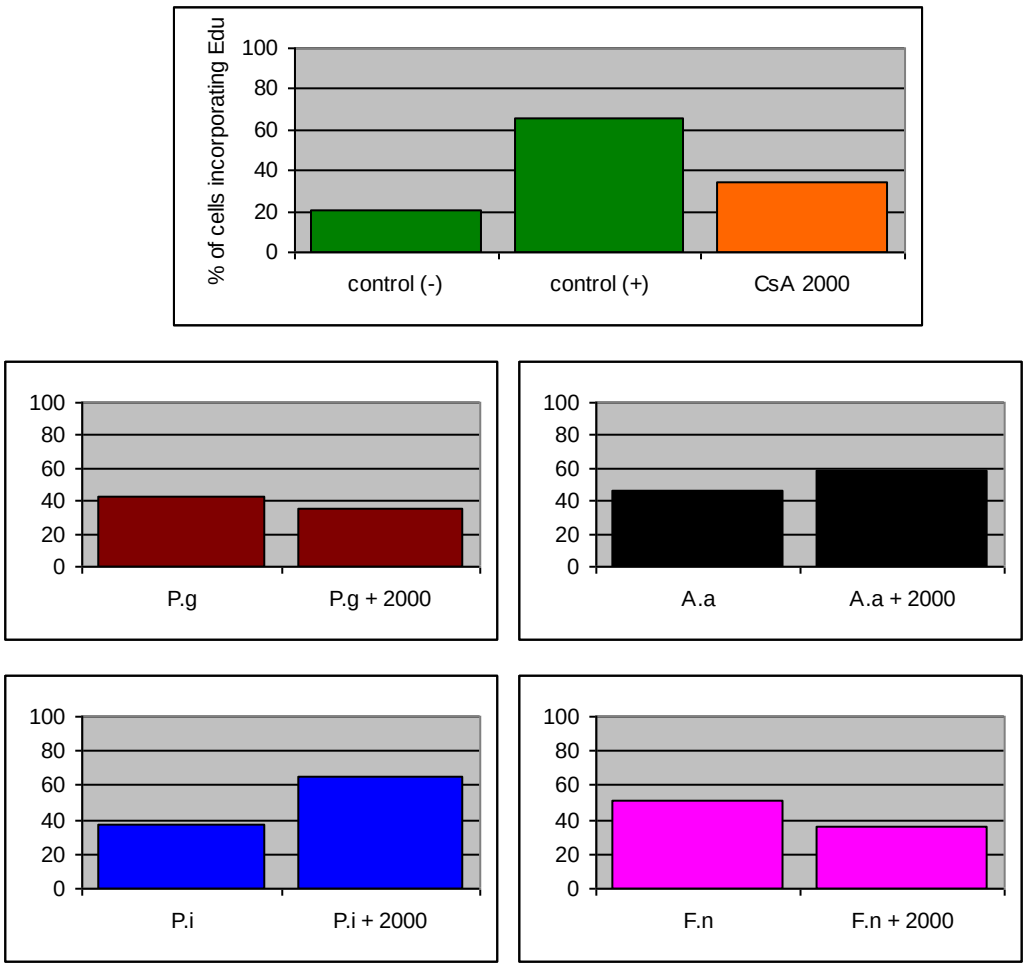


Figure 5.21. **Effect of CsA and bacterial supernatants on proliferation rate of HGF cells**. HGF cells were stimulated for 24h with CsA and bacterial supernatant and proliferation rate assessed by FACS analysis using Edu incorporation. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % of cells incorporating Edu

5.6.3 Cyclin D1 expression:

The addition of serum and CsA increased Cyclin D1 expression (63.7% & 56% respectively) compared to serum free controls (43.5%, Fig 5.22) & (Fig A9, Appendix 2).

P. gingivalis

P.g increased Cyclin D expression (65.1%). This was not affected by the addition of CsA.

A. actinomycetemcomitans

A.a increased Cyclin D expression (62.7%). This was not affected by the addition of CsA.

P. intermedia

P.i increased Cyclin D expression (65.8%). This was not affected by the addition of CsA.

F. nucleatum

F.n increased Cyclin D expression (64.2%). This was not affected by the addition of CsA.

It is evident from the bivariate dot plot analyzing the cyclin D1 expression versus DNA content that the expression of cyclin D1 was mainly found in cells in G1 and early S phases in most HGF cultures (Fig 5.23).

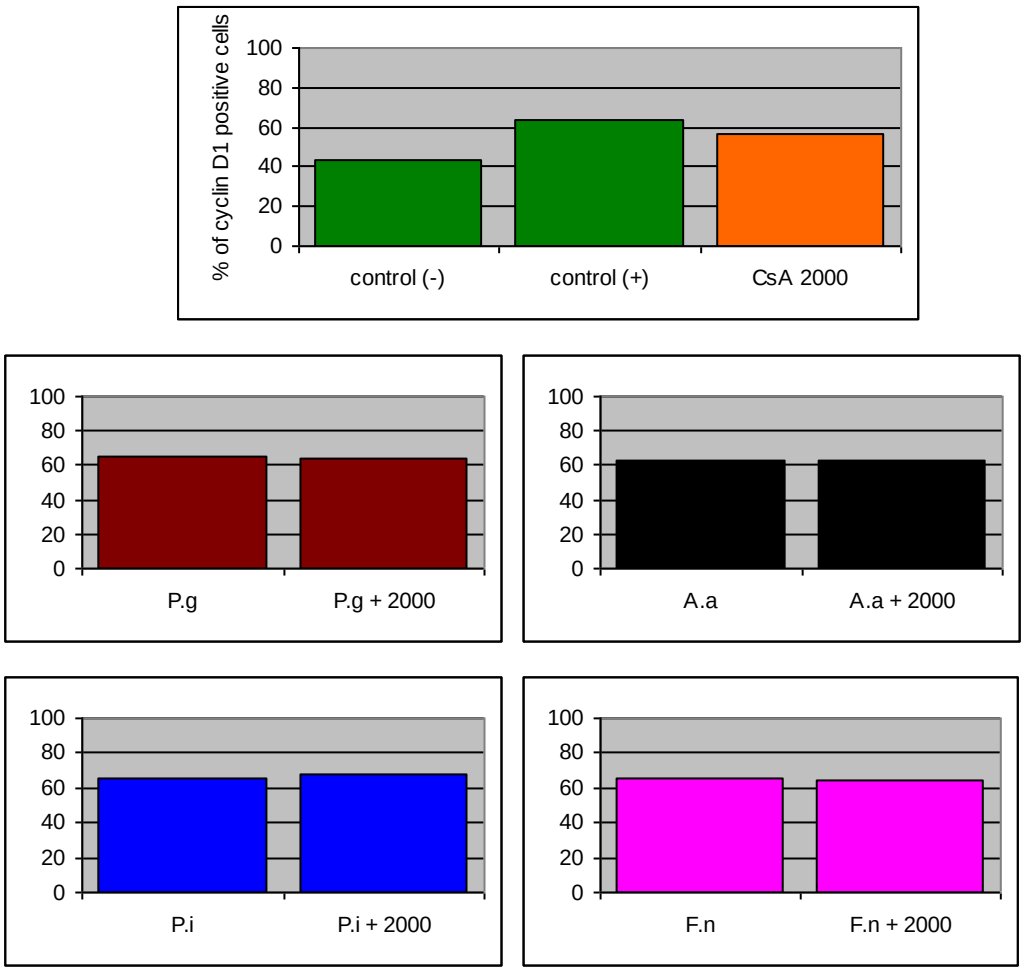


Figure 5.22. Cyclin D1 expression by HGF exposed to a combination of CsA and bacterial supernatants for 24h. HGF cells were stimulated for 24h with CsA and bacterial supernatant and Cyclin D1 assessed by FACS analysis. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % of cyclin D1 positive cells

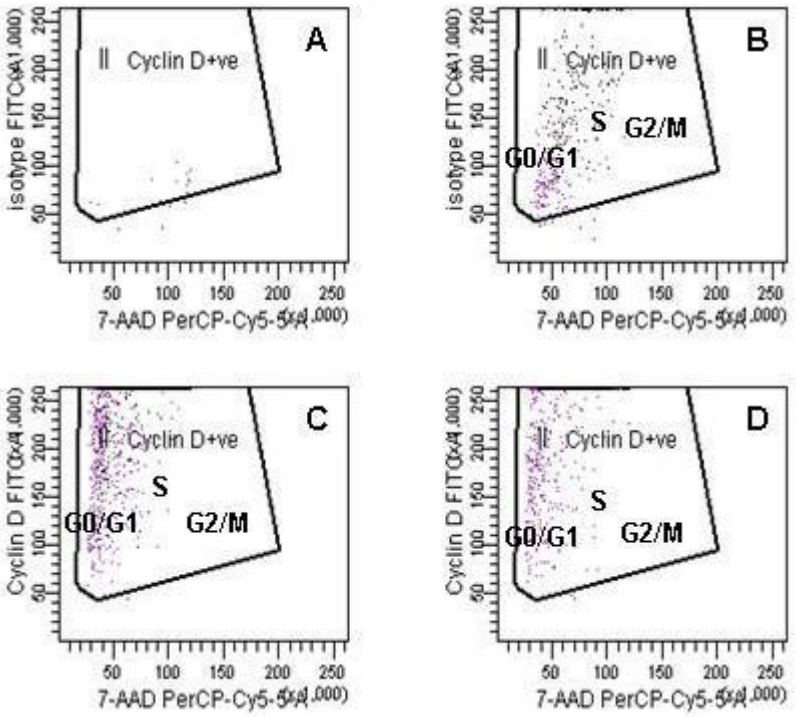


Figure 5.23. Bivariate distributions showing the expression of cyclin D1 in relation to the cell cycle position in HGF cells after 24h exposure to a combination of CsA plus bacterial supernatants. HGF cells were stimulated for 24h with CsA and bacterial supernatant and fashion of Cyclin D1 expression was assessed by FACS analysis. Dual parameter dot plots analysing the cyclin D1 positive cells against DNA content were shown. These dot plots show that cyclin D1 was expressed throughout the cell cycle phases. A)- Isotype control was used to determine the positive and negative populations. B)- serum-starved control. C) HGF cells exposed to P.g supernatant. D)- HGF cells exposed to a combination of P.g supernatant plus 2000ng/ml CsA.

5.6.4 Cyclin B1 expression:

Cyclin B1 was not detected in any of the HGF cultures

5.6.5 Bax expression:

Bax expression was not present in control cells (Fig 5.24). CsA, however increased Bax expression to 5-6% (Fig A14, Appendix 2).

P. gingivalis

P.g increased Bax expression (6%) whilst the addition of CsA abrogated the effect.

A. actinomycetemcomitans

A.a caused a slight increased Bax expression (1%) which was augmented with 250ng/ml CsA (4%). 2000ng/ml CsA reduced Bax expression to below detectable levels

P. intermedia

P.i increased Bax expression (9%) whilst the addition of CsA reduced the effect.

F. nucleatum

F.n treated cells had very low levels of Bax expression, which was increased to 5% by the addition of 250ng/ml CsA. 2000ng/ml CsA reduced Bax expression to below detectable levels

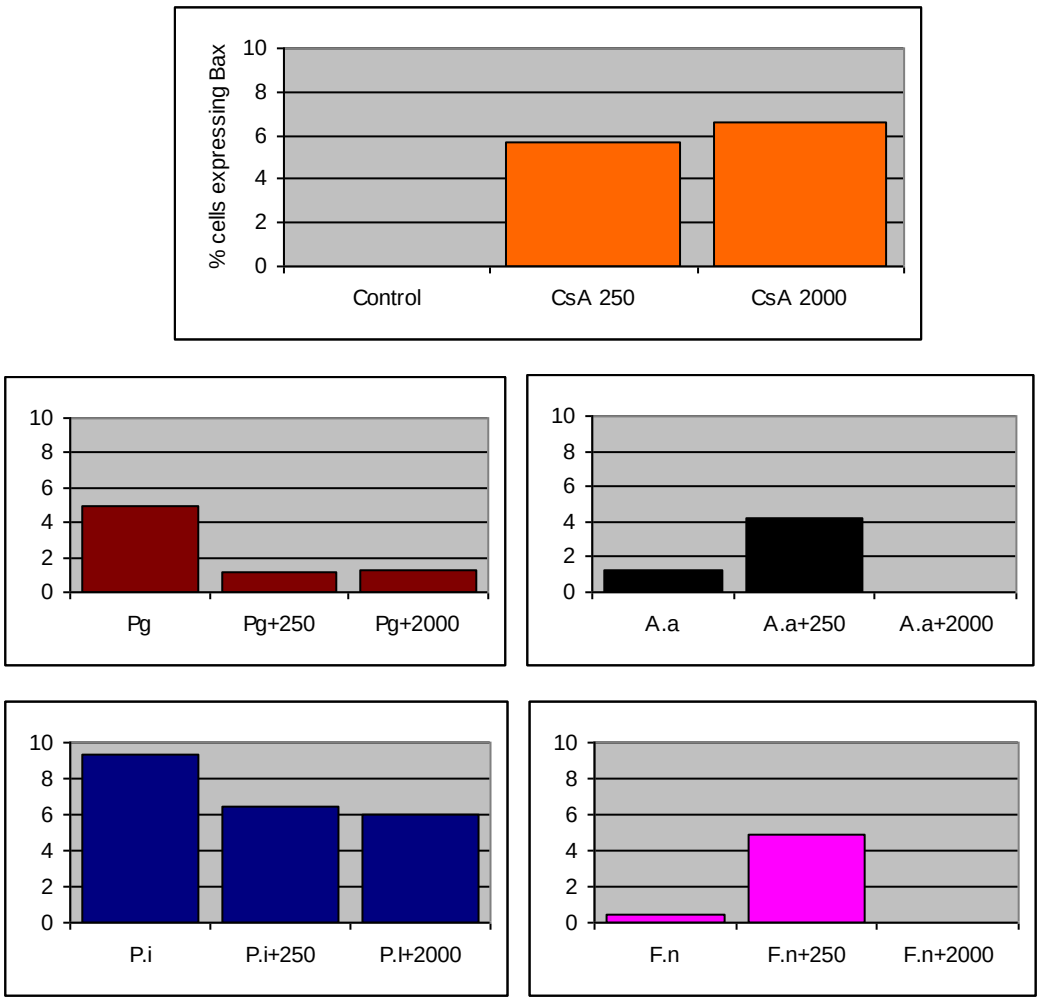


Figure 5.24. Bax expression by HGF exposed to a combination of CsA and bacterial supernatants for 24h. HGF cells were stimulated for 24h with CsA and bacterial supernatant and Bax assessed by FACS analysis. Results are expressed as a proportion of the total number of cells.
X axis represents treatment and Y axis represents % cells expressing Bax

5.6.6 Bcl-2 expression:

Bcl-2 was not detected in any of HGF cultures.

5.7 Summary:

The addition of serum to FIBS, OKF6 and TR146 cells increased the proportion of cells in S and G2/M, whilst 250ng/ml and 2000ng/ml CsA had little effect on cell cycle distribution. Of the bacterial supernatants examined, A.a consistently caused an apparent increase in G2/M fraction in the keratinocyte cell lines studied. This was accompanied by a decrease in G0/G1 and no change in S phase. A.a also increased the proliferation of all the keratinocytes. HGF cells demonstrated a different response with all bacterial supernatants increasing the proportion of S phase fraction without a change in G2/M. A similar increase was also found in TR146 cells exposed to any of the bacterial products.

The combination of CsA with bacterial supernatants had a little effect on cell cycle distribution but caused a limited effect on proliferation rate. P.i + 2000ng/ml CsA increased proliferation FIBS, OKF6 and HGF cells, all combinations of 2000ng/ml CsA and bacterial products inhibited the proliferation rate of TR146 cells. This late effect was associated with abrogating the cell cycle as shown with some combinations.

Unfortunately, the expression level of cyclin D1 in our cell lines did not serve as a marker of the proliferative activity but, interestingly two cell type-dependent patterns of its expression were recognized; whilst its expression was recognized throughout the cell cycle phases in OKF6 and FIBS keratinocytes, an exclusive expression to G1 phase was found in HGF and TR146. This variance in the pattern of cyclin D1 expression, which normally peaks in end of G1 phase, has also been reported in other studies where it was related to the cell type and the transformation phenotype (Collecchi *et al.,* 2000; Darzynkiewics *et al.,* 1996). However, the biological explanation of the unscheduled expression of this cyclin is still unclear but it has been suggested to be a common event in transformed cell lines (Collecchi *et al.,* 2000). In contrast to Cyclin D1, Cyclin B1 was not detected in all cultures studied.

The baseline level of Bax expression ranged from undetectable to very low amounts (0-6%). CsA alone induced very low levels of Bax expression in HGF cells (5-6%) but had no effect on all keratinocyte cells. Induction of Bax expression was frequently found in all keratinocyte cultures exposed to A.a supernatant. Combinations of A.a and 2000ng/ml CsA, however, caused a decrease in OKF6 cells and an increase in TR146. However, all bacterial supernatants induced Bax expression in HGF cells with a more pronounced effect with P.i. Addition of CsA caused a CsA dose and bacterial type-dependent effect on Bax expression in HGF; an increase was found when 250ng/ml CsA was added to A.a or F.n but a decrease was found when CsA (2000ng/ml and 250ng/ml) was added to P.g or P.i. Bcl-2 was not detected in any of cultures studied.

Chapter Six

Materials and Methods (reconstructed epithelial models)

6.1 Introduction:

In the first part of the study the effect of CsA and bacterial products on human keratinocytes and gingival fibroblasts was examined using a monolayer culture system. Keratinocytes, i*n vivo,* however, are present as part of a stratified epithelium and it is possible that this may affect the interaction with CsA and bacterial products. The aim, therefore of this next phase was to evaluate the effect of CsA and bacterial products on an *in vitro* reconstructed model of human gingival and oral epithelium. Tissue viability IL-1α, IL-6, and IL-8 release were assessed using MTT assay and ELISA.

effects on proliferation and apoptosis were also evaluated by examining the expression of proliferation- related genes such as CCNB1 (Cyclin B1) and CCND1 (Cyclin D1), , and the anti-apoptotic gene; B-cell CLL/lymphoma 2 (Bcl2), the gene encoding Bcl-2 protein, as well as pro-apoptotic genes such as Bcl2-associated X protein (Bax) and Fas ligand (Fas-L) that encode Bax and Fas-L proteins respectively.

6.2. Mucosal models:

Two types of reconstructed *in vitro* human epithelial models developed and supplied by SkinEthic Laboratories (Nice, France) were used in this study;

- Reconstructed human oral epithelium (RHOE): This three-dimensional model of the human buccal epithelium has been developed using a transformed oral keratinocyte (TR146) cell line derived from a squamous cell carcinoma of the buccal mucosa (Rupniak et al, 1985).

- Reconstructed human gingival epithelium (RHGE): This three-dimensional model is derived from normal human gingival keratinocytes.

Epithelial cells were cultured on a 0.5cm^2 inert polycarbonate filter raised to the air-liquid interface in a serum free, chemically defined growth medium for 12 days (TR146) and 5 days (human gingival keratinocytes) to form a non-keratinized stratified epithelium with 10-12 cell layers (Buccal phenotype) or a keratinized

stratified epithelium (Gingival phenotype). (Rosdy & Clauss, 1990; Rosdy *et al.*, 1993). (Figs 6.1 - 6.3).

SkinEthic laboratories also provided growth medium MCDB 153 containing 5µg/ml insulin, 1.5mM CaCl2, 25µg gentamycin and 0.4 µg hydrocortisone for use in the experiments.

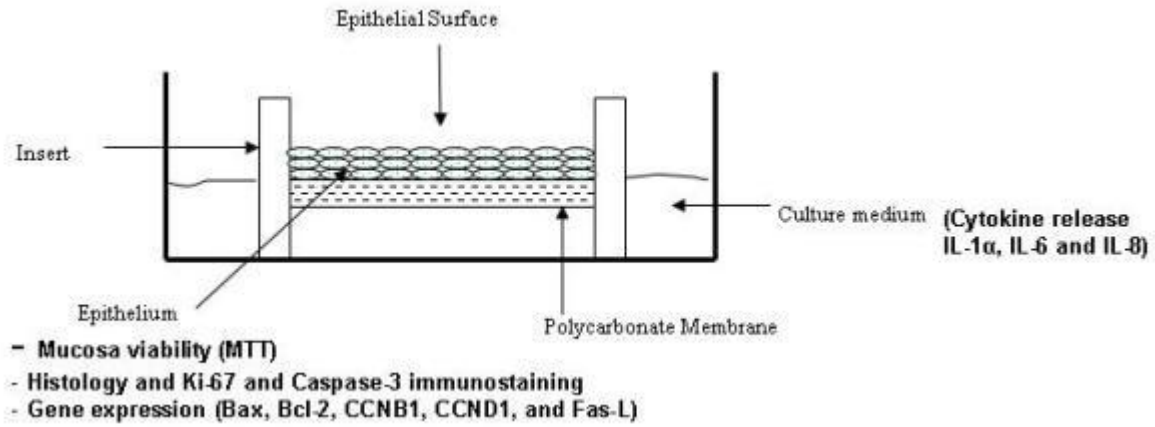


Figure. 6.1. Diagram of Reconstituted Mucosal Model (Courtesy of Dr A. T. Cruchley)

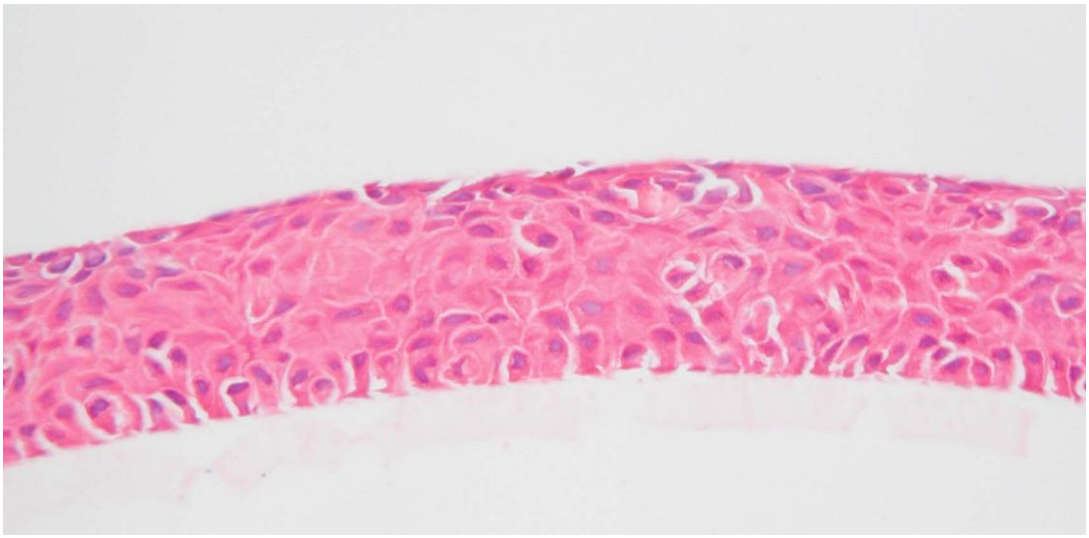


Figure. 6.2. Light micrograph of uninfected control tissue of reconstituted human buccal mucosa stained with haematoxylin & eosin. Original magnification x 63.

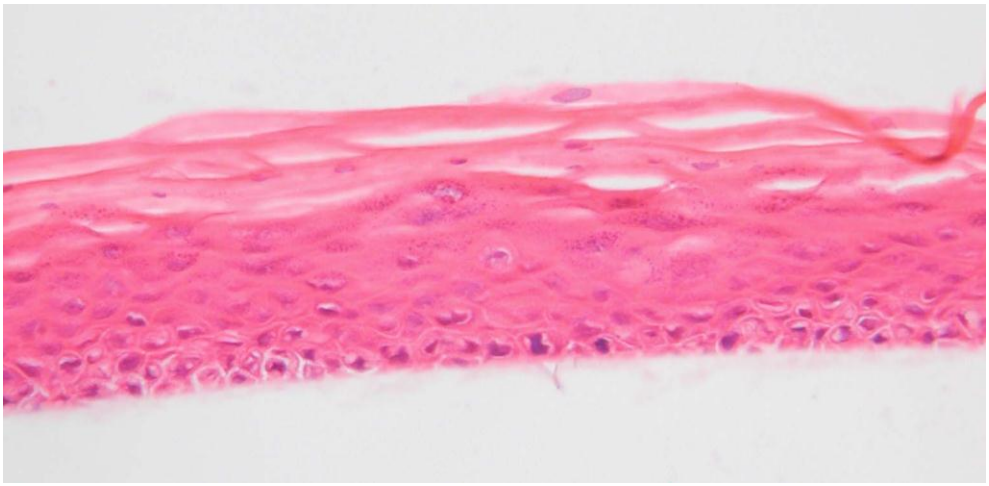


Figure. 6.3. Light micrograph of uninfected control tissue of reconstituted human gingiva stained with haematoxylin & eosin. Original magnification x 63.

6.3. Treatment:

The mucosal cultures were shipped on agarose gel and, on arrival, were transferred into 24 well-plates pre-filled with 0.5 ml growth medium (GM). 50µl PBS (control) or bacterial supernatants (Table 6.1) was applied to the epithelial surface and the cultures were incubated overnight at 37^{o}C in 5%CO_2 in a humidified atmosphere.

Bacteria	Dilution (in PBS)
P. gingivalis	1/10
A. actinomycetemcomitans	1/10
P. intermedia	1/10
F. nucleatum	1/10

Table 6.1. Bacterial supernatants used in the study.

6.4. Experimental design:

Cultures, pre-exposed to PBS or bacterial supernatants, were washed by gently pipetting 200µl PBS (3 times in 5 minutes) and transferred into 6 well-plates containing 2 ml GM supplemented with 0 (control) or 2000 ng/ml CsA (baseline). Fresh PBS or bacterial supernatant was then re-applied to the epithelial surface and the cultures were then incubated for up to 48hrs at 37^{o}C and 5%CO_2. Culture medium was collected at 6h, 24h, and 48h after the baseline point and stored at -80^{o}C until used to assess cytokine and growth factor release. At each time point the culture medium was replaced and the epithelium was washed and re-challenged with PBS or CsA or/and bacterial supernatant.

In addition to cytokine and growth factor release, epithelial viability was assessed using the MTT assay on triplicate samples of the epithelium at 48hrs. In addition, one culture was used for RNA extraction for qPCR, and one culture fixed in neutral buffered formalin for morphological and immunohistochemical investigation. Controls included cultures stimulated with 0ng/ml CsA and exposed to PBS only as a negative control, bacterial supernatant-exposed cultures without addition of CsA, and PBS-exposed cultures with addition of CsA.

6.5. Epithelial viability:

At the end of the treatment period, the cultures were transferred to a 24-well plate containing 500µl of MTT solution (0.5mg/ml 3-(4,5dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide in PBS). The plate was wrapped in aluminium foil and incubated for 1 hour at 37ºC in 5% CO_2 in a humidified atmosphere. After incubation, the cultures were transferred to a new 24-well plate containing 750µl isopropyl alcohol per well and 750µl of isopropyl alcohol applied to the epithelial surface. The plate was carefully sealed with parafilm, to prevent evaporation, and then incubated at 37ºC for 1 hour to extract the formazan. After 1 hour the membrane was punctured, expended cultures discarded and the isopropyl alcohol mixed in order to equilibrate the colour density.

The optical density (OD) was measured at 570nm using an Optima plate reader. Tissue viability was expressed as:

Tissue viability = OD (570nm) Test/ OD (570nm) control x 100.

6.6. Cytokine release:

IL-1α, IL-6, and IL-8 release from RHGE and RHOE exposed to a combination of CsA along with bacterial products was determined by sandwich Elisa (Duo Set®; R & D systems, Abingdon, UK) according to the protocol outlined in chapter 2.

6.7. RNA extraction:

The epithelium was dissected from the insert using a clean, sterile scalpel and immediately immersed in 500 µl RNA Later solution (Qiagen, Hilden, Germany) and then stored at -80^{o}C until use for RNA extraction.

Total RNA was extracted from mucosal samples using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacture's instructions with simple modifications as follows:

1. Defrost samples at room temperature.
2. Remove RNA- Later with 1ml pipette; add 150µl RNeasy lysis buffer RLT (containing 1% β-mercaptoethanol) and vortex to ensure complete lysis.
3. Transfer the lysate to QIAshredder spin column and homogenize by centrifugation for 2 minutes at maximum speed.
4. Transfer the lysate to DNA genome - eliminator column and centrifuge for 30 second at 7000 rpm.
5. Add 150 µl of 70% ethanol to the lysate and mix well by pipetting up and down.
6. Transfer the mixture into a RNeasy spin column and centrifuge for 15 seconds in 7000 rpm. Discard the flow-through.

7. Add 700µl RNeasy wash buffer (RW1) to the RNeasy spin column and centrifuge for 15 seconds in 7000rpm for washing purpose. Discard the flow-through.
8. Wash the RNeasy spin column with 500µl RPE buffer and centrifuge for 15 seconds at 7000rpm for washing purpose. Discard the flow-through
9. Wash the RNeasy spin column with further 500 µl RPE buffer and centrifuge for further 2 minutes at 7000 rpm to dry membrane. Discard the collection tube including the flow-throw.
10. Place RNeasy spin column on a new 2ml collection tube and then centrifuge for one minute at max speed to eliminate residual ethanol.
11. Transfer the RNeasy spin column to a new 1.5 ml collection tube and elute the RNA bound to the membrane with 30 µl RNase-free water with waiting time 10 minutes and then centrifuge for 1 minute in 7000rpm to collect total RNA.
12. Collect the eluate obtained in the last step and re pipette onto the membrane to re-elute any remaining RNA that might still be bound to the RNeasy spin column membrane with waiting time 10 minute and then centrifuge for further 1 minute in 7000 rpm.

Finally, discard the RNeasy spin column and measure the concentration of extracted RNA by NanoDrop (NanoDrop® ND-1000, Spectrophotometer Labtech International, UK) at wavelength of 260nm. Determine the purity of RNA by the 260/280nm ratio which should be within range of 1.9 to 2.1. Store the extracted RNA at -80^{o}C until use.

6. 8. Reverse Transcription (RT):

Since RNA can not be used as a template for PCR, the first step in an RT-PCR assay is the reverse transcription of the RNA into first strand cDNA. The reverse transcription system kit (Catalogue No: A3500, Promega Corporation, Madison, Wisconsin USA) was used for cDNA synthesis from total RNA extracted from mucosal cultures in two steps according to manufacture's instructions.

The primer composed of (0.5µl Oligo dT and 0.5µl random primer) was added to 13.5 µl total volume of 2µg total RNA in nuclease-free water in 0.5 ml PCR tubes,

incubated at 70^{o}C for 5 minutes, and subsequently chilled rapidly on ice and centrifuged briefly to collect the condensation.

1. Reverse transcription was achieved by the addition of 12.25µl total volume of RT reaction master mix prepared at the final concentration of 5µl of $MgCl_2$ (25mM), 2.5µl of 10X buffer, 2.5µl of dNTP, 2.5µl of RNasin, and 0.5µl of AMVRT.
2. The mixture was then vortexed for 3 seconds, centrifuged briefly to collect residual liquid from the walls of the tubes, and incubated in the PCR thermocycler (Thermal cycling system, HYBAID, Middlesex, UK) at 42^{o}C for 30 minutes, heated to 99^{o}C for 3 minutes, and then cooled down to 4^{o}C.
3. At the end of the RT reaction, the yielded cDNA products were diluted in 50µl nuclease-free water (Promega Corporation, Madison, Wisconsin USA) to give a final total volume of 75 µl. cDNA and stored at -80^{o}C until PCR amplification.

6.9. Absolute Real time-quantitative PCR using SYBR Green I dye:

6.9.1. Principle:

Quantitative PCR is a method being used to simultaneously quantify and amplify a target DNA molecule. The SYBR Green system used in our study is based on a principle of binding of the SYBR-Green I dye to all double-stranded DNA in a PCR reaction that evokes fluorescence with intensity proportional to the concentration of DNA product. Hence, measurement of the fluorescence intensity generated at each cycle allows quantification of DNA concentrations (Arya *et al.,* 2005).

6.9.2. Primer design:

The PCR primers were designed using the *http//www.roche-applied science.com/sis/rtpcr/upl/adc.jsp* software from gene sequences obtained from the National Centre for Biotechnology Information database (www.ncbi.nlm.nih.gov). Primers were selected to have approximately the same melting temperature (Tm)

ranging from 58 to 60^{o}C and length of amplification product to be around 100bp. Primers were purchased from Sigma (Table 6.2).

Table 6.2. Primer sequences used in the study

Gene	Sequence	Accession No	Size (bP)
Bax	F: agcaaactggtgctcaagg R: gctgaggcaggtgaatcg	NM_138764.3	93
Bcl-2	F: gcacctgcacacctggat R: agccaggagaaatcaaacagag	NM_000633.2	96
Cyclin B1	F: catggtgcactttcctcctt R: aggtaatgttgtagagttggtgtcc	NM_031966.2	102
Cyclin D1	F: tcacacgcttcctctccag R: tggggtccatgttctgct	NM_053056.2	120
Fas-L	F: gttctggttgccttggtagg R: tgtgcatctggctggtagac	NM_000639.1	100
POLR2A	F: gcaaattcaccaagagagacg R: cacgtcgacaggaacatcag	NM_000937	73
YAP1	F: cccagatgaacgtcacagc R: gattctctggttcatggctga	NM_001130145	83

6.9.3. Control Reference Genes:

All target gene expression levels were normalized using two reference genes, polymerase (RNA) II (DNA directed) polypeptide A (POLR2A) and Yes-associated protein 1 (YAP1) (Table 6.2), which were previously shown to be stable across various human normal oral mucosa, dysplastic and squamous cell carcinoma cells (Gemenetzidis *et al.*,, 2009).

6.9.4. Construction of standard curve:

In order to quantify the mRNA copy number, a standard curve was performed based on a serial dilution of the PCR product of each gene with a known starting concentration (Gemenetzidis *et al.*,, 2009). A standard curve for each gene studied was generated in two steps. First, a PCR reaction was run for each primer pair to amplify a large amount of PCR product from a pooled cDNA sample (containing

various human oral epithelial cell lines, a generous gift by Dr. Muy-Teck Teh, CDOS) using a SYBR Green 1 Master qPCR kit (Roche Diagnostics Ltd, Lewes, UK). The 50µl final volume for each PCR reaction/per well consisted of; 20µl of fast start SYBR Green 1 master mix, 20µl dH2O, 5µl cDNA template, and 7µl of 5µM forward/reverse primer (0.7 µM final concentration of each primer). PCR amplification was performed using the LightCycler LC480 machine according to the following program:

- An initial denaturation at 95^{o}C for 5 minutes.
- Followed by 40 cycles of 95^{o}C for 10s, annealing at 60^{o}C for 6s, and extension at 72^{o}C for 6s.

At the end of 40 cycles, a melting analysis was performed for each sample to check for a single peak to confirm the primer specificity for each gene. During the melting analysis, samples were heated from 65^{o}C to 99^{o}C at a steady rate of 0.10^{o}C/s, and fluorescence measured continuously throughout this period.

The PCR products were then purified using a DNA purification spin column (Qiagen).

1. Each PCR product was placed onto a Qiagen spin column followed by 250µl PB (Binding Buffer, Lot No 11546581 Qiagen GmbH D-40724, Hilden, Germany) and then centrifuged at 15000 rpm for a maximum of 1 minute.
2. The flow through was discarded and 0.7ml PE wash buffer (wash buffer, Lot No 124106145, Qiagen GmbH D-40724, Hilden, Germany) was then added and the columns were centrifuged at for a further up to 1 minute (maximum) followed by a dry spin for the same time.
3. The bottom column was replaced with a new column and 40µl of H_2O was added to the middle of the filter and DNA was eluted by centrifugation at for 1 minute.
4. The liquid from the bottom columns were collected and placed in pre-labelled tubes.
5. The concentration of the purified PCR products for each gene was subsequently measured using the NanoDrop and the total number of copies in PCR product calculated using the Avogadro's Constant where 1mole = $6.02*10^{23}$ molecules.

A stock solution of 10^{11} copies/2µl for each PCR template was prepared and a dilution series of 10^9, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 DNA copies per 2µl was generated by diluting the stock in tRNA (25µg/ml; Sigma R8508) as a DNA carrier as follows:

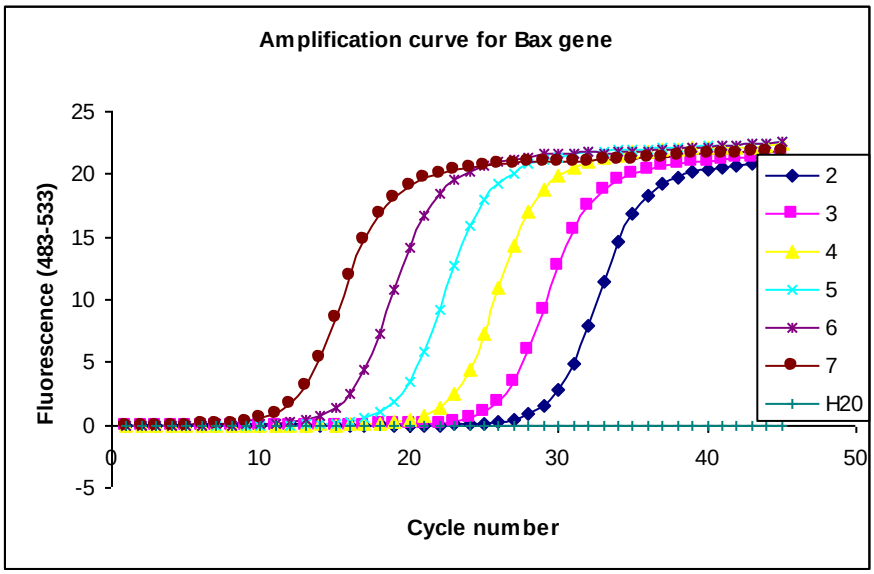
- To make 10^9 from 10^{11}, add 2µl of 10^{11} into 198µl tRNA
- To make 10^7 from 10^9, add 2µl of 10^9 into 198µl tRNA. …and so on.

All standard dilutions were stored at -20^{o}C until use.

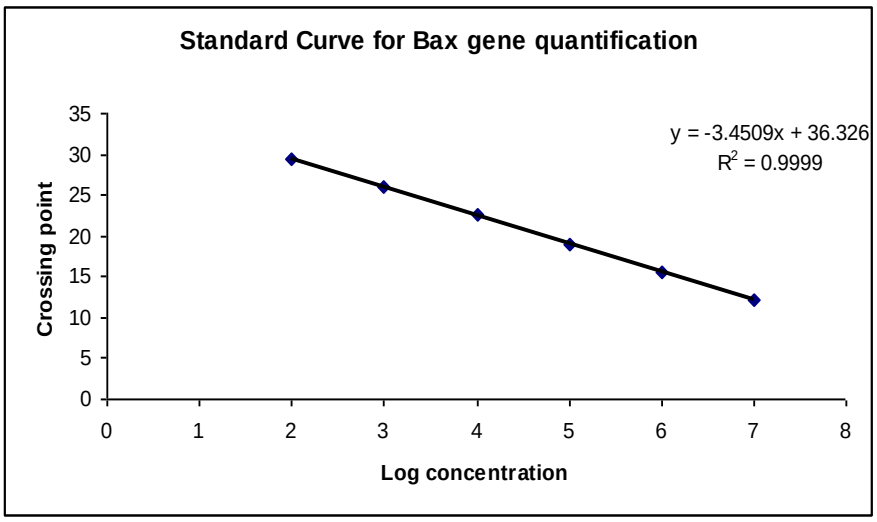
The standard curves for each PCR product was created by plotting the logarithm of the initial copy number of the standard template (X-axis) against the respective cycle number of crossing point (Cp), the point at which the fluorescence increases significantly above the background fluorescence (Y-axis). The PCR amplification efficiency for each gene was then calculated by an internal algorithm within the Roche LightCycler Software system. Subsequently, extrapolating the Cp values of unknown samples to this curve allows quantification of the initial copy numbers of the respective genes. Amplification efficiencies and melting temperatures for the target genes are shown in Table 6.3.

Fig 6.4 displays the LightCycler qPCR amplification plot and the corresponding melting curve and standard curve for Bax gene quantification. The standard curve was linear ($R^2 = 0.99$) within the range tested (from 10^2 to 10^7 copies/µl) by the triplicate reactions.

A


Amplification curve for Bax gene
Fluorescence (483-533)
Cycle number
25
20
15
10
5
0
-5
0
10
20
30
40
50
2
3
4
5
6
7
H20

B


Standard Curve for Bax gene quantification
Crossing point
Log concentration
y = -3.4509x + 36.326
R2 = 0.9999
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8

C

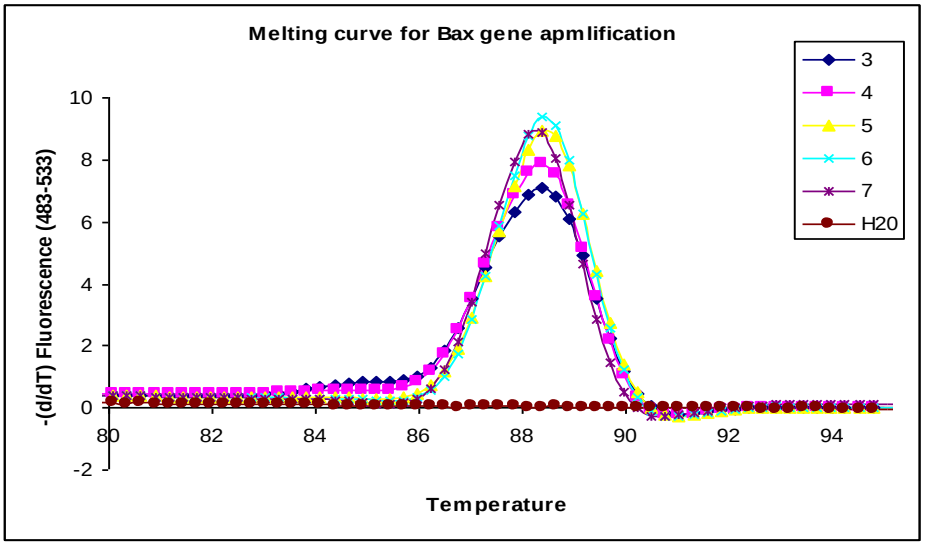


Figure 6.4. LightCycler qPCR amplification reaction for quantification of Bax gene in a standard dilution series using SYBR Green I dye. The standard curve was generated from serial dilutions of PCR product ranging from 10^2 to 10^7 copies/µl.
A- A representative plot of qPCR amplification for Bax gene performed using the LightCycler 480 Machine. All samples were amplified for 45 cycles along with measuring the fluorescence emission at the end of each extension stage. The increase in fluorescence density was plotted versus the PCR cycle number.
B- A representative standard curve for Bax gene was created by plotting the cycle number of crossing point (CP) generated from the amplification plot in A against the input cDNA copy number. Thus, by extrapolating the CP values of the unknown samples to this standard curve, the starting template concentration of the unknown samples can be measured.
C- A representative melting curve analysis from the same amplification reaction. Samples were slowly melted along with continuous measurement of the fluorescence. The melting curve was created by plotting the negative derivative of fluorescence generated from each sample versus the temperature ^{o}C. A single peak was detected at the temperature (88.5^{o}C) confirming the specificity of primers.

Table 6.3. Efficiencies and melting temperatures for qPCR amplifications from the house keeping and target genes.

Gene	Efficiency	Tm (melting peak)
CCND1	1.851	89.13 ± 0.168
CCNB1	1.941	80.65 ± 0.091
Bax	1.932	88.32 ± 0.07
Bcl-2	1.864	85.78 ± 0.17
Fas-L	1.782	84.22 ± 0.1
YAP1	1.948	84.01±0.1
POLR2A	1.970	82.12 ± 0.06

6.9.5. PCR procedure:

Quantitative PCR was conducted using a LightCycler LC480 machine (Roche Diagnostics Ltd, Lewes, UK).

1. Reactions were set up in 96-well optical plates (Roche Diagnostics) using the following final concentrations:

   4µl of Fast start DNA Master SYBR Green 1 mix (containing *Taq* DNA polymerase, dNTP, $MgCl_2$, and SYBR Green I dye); 0.5 µl of each forward and reverse primer (5µM; 0.5 µM final concentration), 4.5 µl of dH2O and 2 µl of cDNA template/DNA standard in a 10 ± 1µl total reaction mixture.
2. Triplicate reactions were run for each sample along with reactions containing RNase-free water instead of DNA template (as negative control) in every qPCR run.
3. The plates were tightly sealed with a Roche sealing film (Cat. NO 04729692001) and spun for 30s at 3000 rpm.
4. PCR reactions were then run in the LightCycler 480 machine according to the following cycling conditions: denaturation and "hot start"(95° C for 5 minutes), 45 cycles of amplification and quantification consisted of (melting for 10s at 95°C, primer annealing at 60°C for 6s and extension/acquisition at 72°C for 6s.
5. A melting curve analysis was then performed to confirm the specificity of the PCR products according to the following program (melting at 95^oC for 30s, cooling/annealing at $65^oC$for 30s, 65^oC to 99°C gradual heating with a heating rate of 0.1° C/s along with continuously measuring the fluorescence and the program was then terminated with a cooling step to 40°C for 10s).
6. Data were analyzed using LightCycler software and gene expression was then normalized to the reference genes (POLR2A and YAP1) and subsequently normalized to the biologic control (un-treated sample).

6.9.6. Agarose gel electrophoresis:

In order to confirm the specificity of primers used, the corresponding amplified PCR products were confirmed to be single band at the predicted size using agarose gel electrophoresis.

Briefly, 2% agarose (Melford Ltd. Ipswich. UK) was totally dissolved in 50ml 1x TAE by microwaving at max power for 3*30 second. The solution was then allowed to cool on the bench for 5 minutes, 1µl of ethidium bromide was afterwards added and dispersed into the solution by stirring, the gel was poured into the tank and bubbles were pushed away to the side, the comb was inserted and the gel was allowed to solidify for 40 minutes, the tank was then filled with 1x TAE. Afterwards, the samples were prepared by mixing 5µl of PCR product with 2µl of loading buffer (Invitrogen.Uk), 7µl of each sample and a DNA hyperladder V (Bioline. London. UK) was loaded into the appropriate wells, electrophoresis was run at 100V for about 50 minutes. Finally, the DNA bands were visualized and photographed by using an ultraviolet transilluminator.

6.10. Data Analysis:

Data were analysed by using One-way ANOVA test along with Student's independent t-test. Statistical significance was considered when $p<0.05$. Findings were further confirmed using SPSS program by one-way ANOVA and Post hoc test.

Chapter Seven

Effect of CsA and bacterial supernatants on reconstructed epithelial models

7.1 Introduction:

This chapter shows the effect of 2000ng/ml CsA along with bacterial supernatants on an *in vitro* reconstructed model of human gingival and oral epithelium in terms of tissue viability, cytokine and chemokine release (IL-1α, IL-6, and IL-8), expression of proliferation-related genes (CCNB1 and CCND1), and apoptosis-related genes (Bax, Bcl-2, and Fas-L).

7.2 Effect of CsA and bacterial supernatants on reconstructed human gingival epithelial cultures (RHGE):

7.2.1 Tissue Viability:

CsA alone had no significant effect on RHGE viability compared to the control (CsA; 92.6 ± 10.8 vs. control; 100 ± 6%; $p>0.05$; Fig 7.1).

P. gingivalis

P.g caused a significant reduction in the tissue viability (68.4 ± 3.3%; $p<0.05$) compared to the control whilst the addition of CsA reduced this effect with viability returning to control levels (93 ± 7% $p<0.05$)

A. actinomycetemcomitans

A.a caused a significant reduction in the tissue viability (74.4 ± 5.6%; $p<0.05$) compared to the control whilst the addition of CsA reduced this effect with viability returning to control levels (90 ± 5.4%; $p <0.05$).

P. intermedia

P.i caused a significant reduction in the tissue viability (66.9 ± 4.8%; $p<0.05$) compared to the control whilst the addition of CsA caused a slight reduction in this effect (82.5 ± 5.8%, $p<0.05$)

F. nucleatum

F.n had no effect on tissue viability, although the subsequent addition of CsA caused a significant increase in tissue viability compared to controls (117.2 ± 1.8%, $p<0.05$ Fig 7.1).

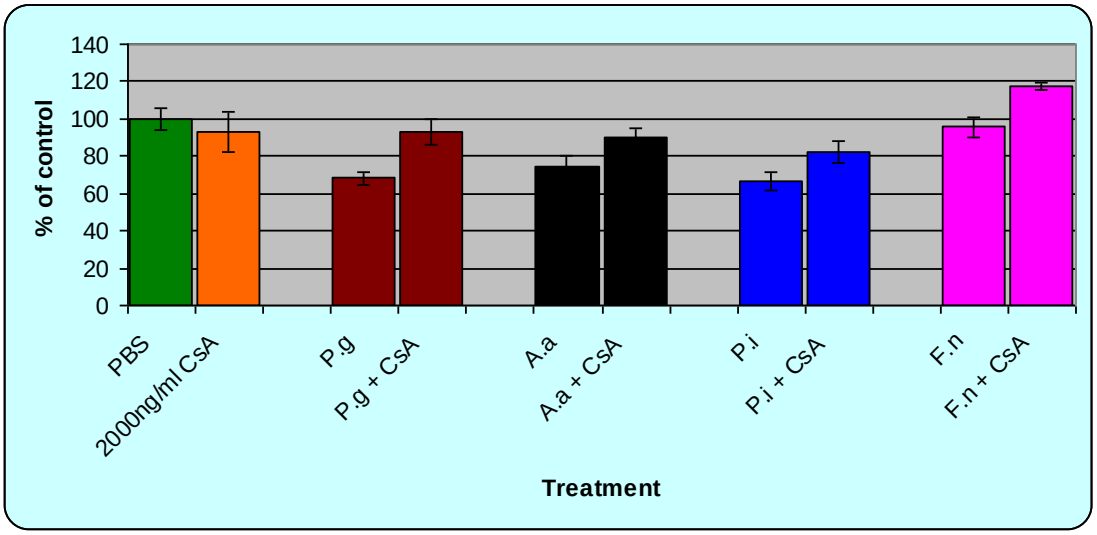


Figure 7.1. Effect of CsA and bacterial supernatants on RHGE viability.
RHGE cultures were stimulated with 2000ng/ml CsA or/and bacterial supernatant. Cell viability was measured by MTT assay after 48h exposure. Control cultures were exposed to PBS. Results are expressed as the mean ± SEM (n=3). Statistical significance was regarded when $p < 0.05$ compared to control.

7.2.2 IL-1α release:

CsA alone had no significant effect on IL-1α release of compared to the control up to 48h (Fig 7.2)

P.g, A.a. P.i, and F.n alone or in combination with CsA had no significant effect on IL-1α release up to 48h (Fig 7.2).

7.2.3 IL-6 release:

CsA alone had no significant effect on IL-6 release compared to the control up to 48 h (Fig 7.3).

P.g, A.a, P.i, and F.n alone or in combination with CsA had no significant effect on IL-6 release up to 48h (Fig 7.3).

7.2.4 IL-8 release

RHGE alone or in combination with CsA released low levels of IL-8 up to 48h (control; 3 ± 0.7pg/ml & 2 ± 0.3 pg/ml respectively; Fig 7.4)

P. gingivalis

P.g had no effect on IL-8 release although the addition of CsA caused a significant reduction in IL-8 release (P.g + CsA; 2.4 ± 0.2 & 1.2 ± 0.2pg/ml at 24h & 48h respectively; $p<0.05$ vs. control).

A. actinomycetemcomitans

A.a caused a significant increase in the IL-8 release after 24 and 48h (A.a; 12 ± 2.9 & 5.4 ± 1.1pg/ml respectively; $p<0.05$ vs. control & CsA). CsA significantly reduced this effect with values returning to controls levels (3.3 ± 0.6 & 1.7 ± 0.2pg/ml at 24h & 48h respectively; $p<0.05$)

P. intermedia

P.i caused a significant increase in the IL-8 release after 24 and 48h (14.4 ± 2.3pg/ml & 5.1 ± 0.4 pg/ml respectively) CsA significantly reduced this effect with values returning to control levels 3.1 ± 0.73 & 1.9 ± 0.6 pg/ml at 24h & 48h respectively; $p<0.05$).

F. nucleatum

F.n alone or in combination with CsA had no significant effect on IL-8 release.

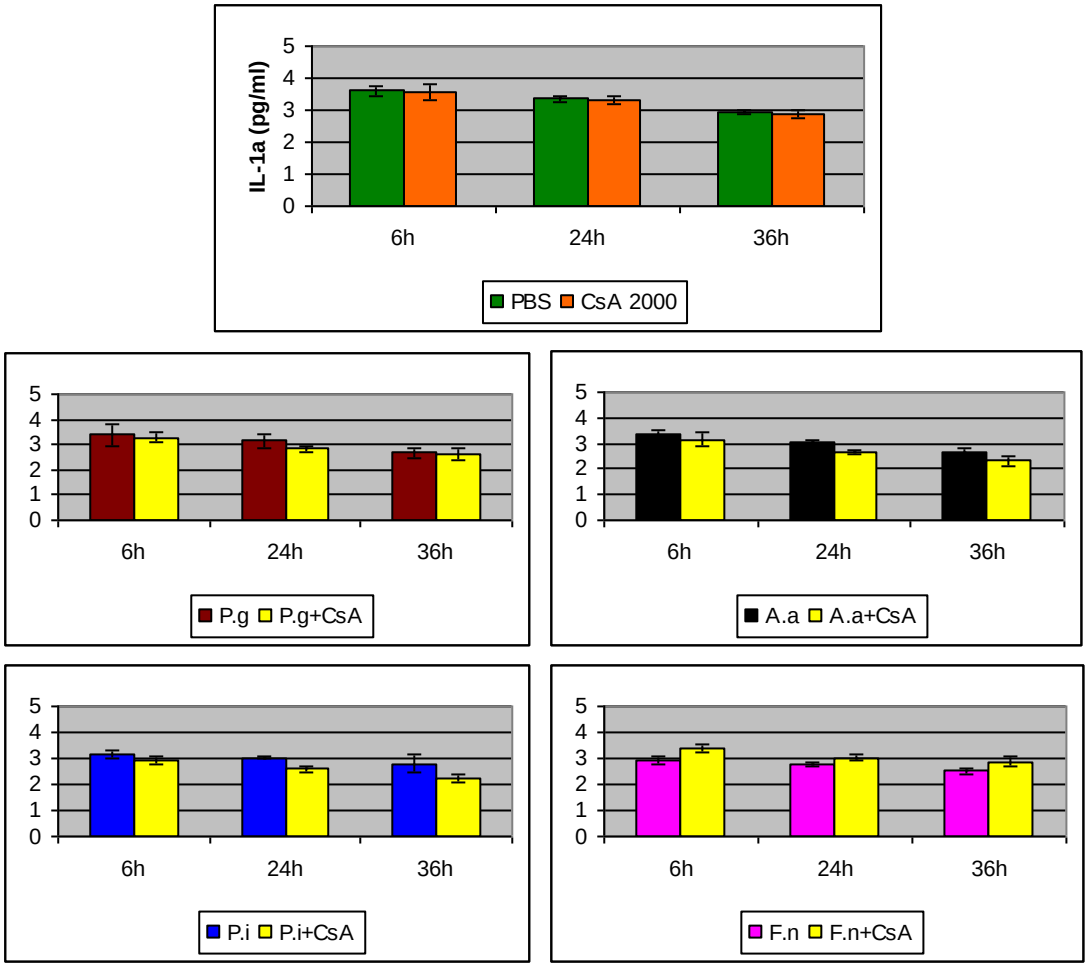


Figure 7.2. Effect of CsA/CsA+ bacterial supernatant combinations on release of IL-1α in RHGE. RHGE cultures were stimulated with 2000ng/ml CsA and/or bacterial supernatant. Control cultures were exposed to PBS. IL-1α was measured by Elisa assay after 6h, 24h, and 48h exposure. Results are expressed as the mean ± SEM (n=5). Statistical significance was regarded when $p < 0.05$ compared to control. X axis represents treatment time (hours). Y represents concentration of cytokine (pg/ml).

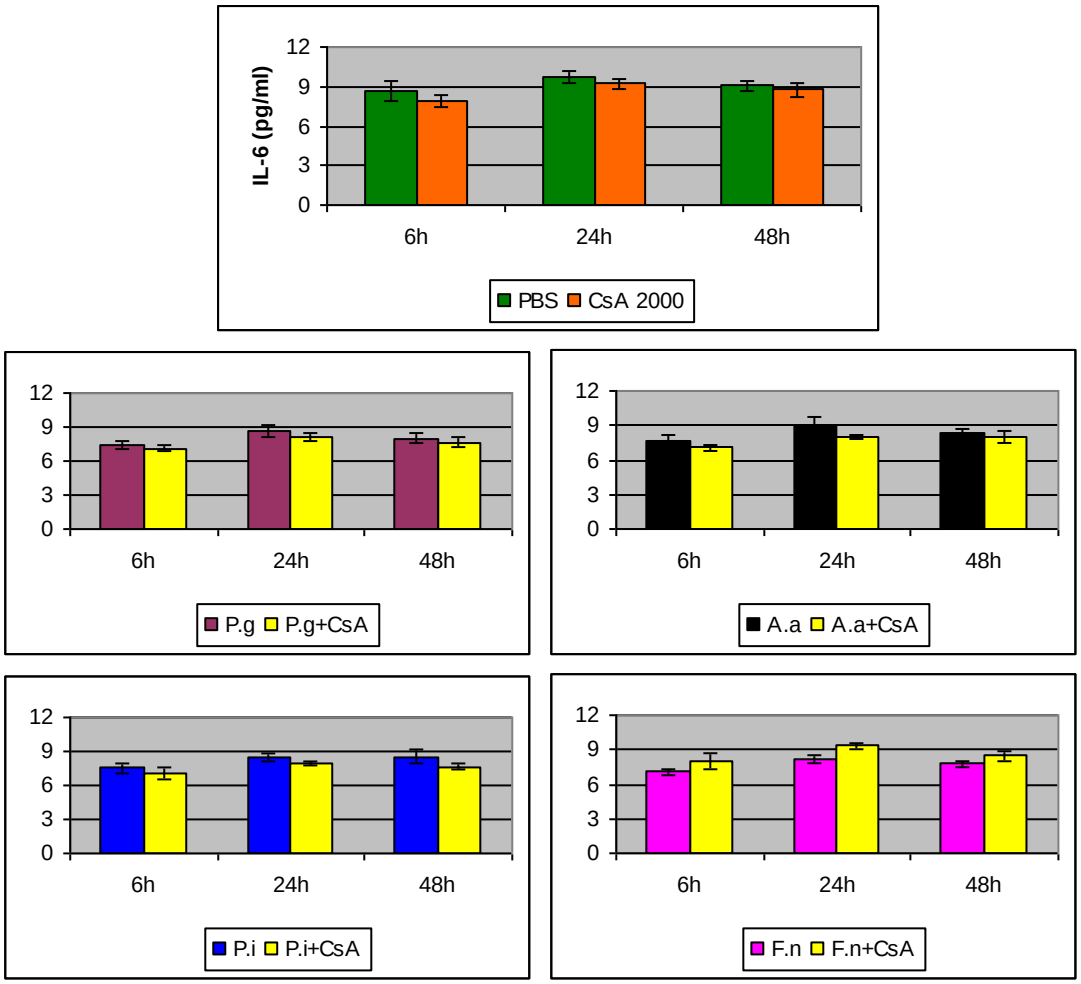


Figure 7.3. Effect of CsA/CsA+bacterial supernatant combinations on release of IL-6 in RHGE. RHGE cultures were stimulated with 2000ng/ml CsA and/or bacterial supernatant. Control cultures were exposed to PBS. IL-6 was measured by Elisa assay after 6h, 24h, and 48h exposure. Results are expressed as the mean ± SEM (n=5). Statistical significance was regarded when $p < 0.05$ compared to control. X axis represents treatment time (hours). Y represents concentration of cytokine (pg/ml).

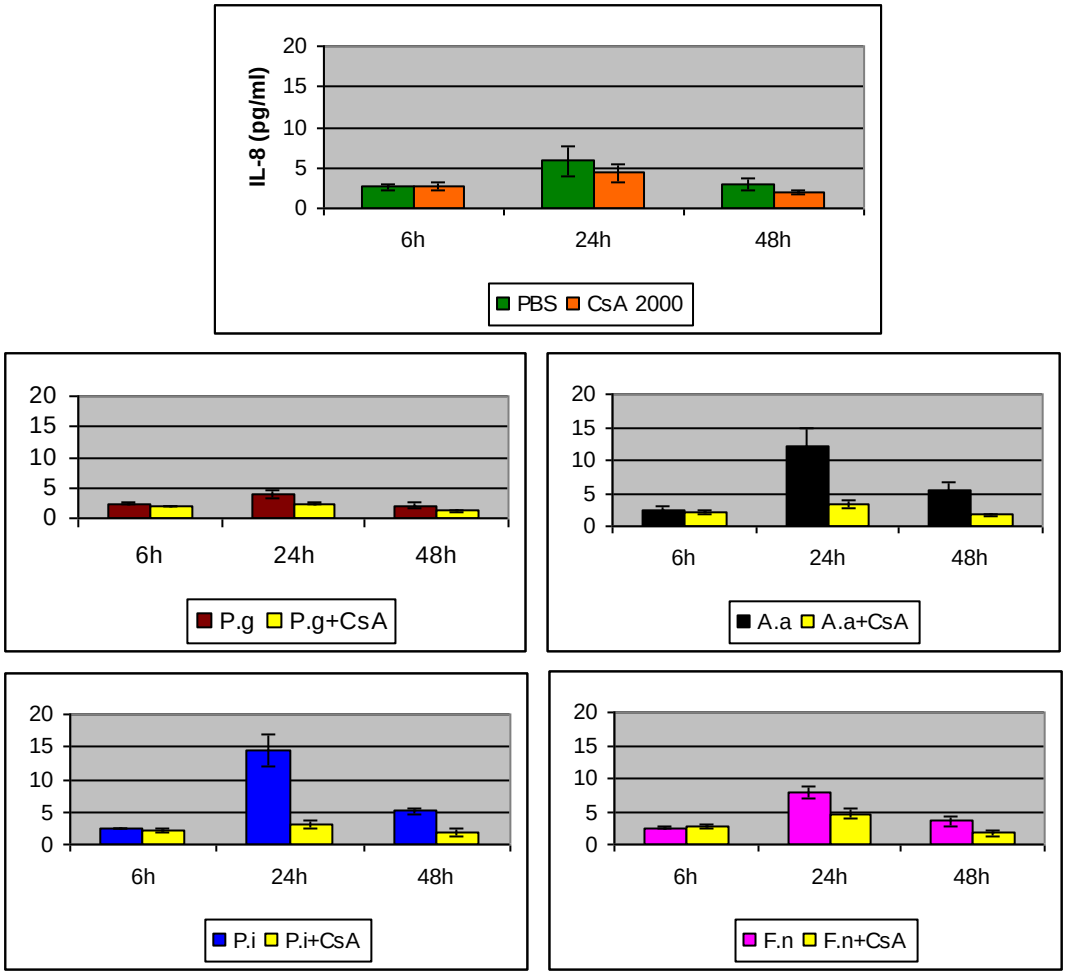


Figure 7.4. Effect of CsA/CsA+bacterial supernatant combinations on release of IL-8 in RHGE. RHGE cultures were stimulated with 2000ng/ml CsA and/or bacterial supernatant. Control cultures were exposed to PBS. IL-8 was measured by Elisa assay after 6h, 24h, and 48h exposure. Results are expressed as the mean ± SEM (n=5). Statistical significance was regarded when $p < 0.05$ compared to control. X axis represents treatment time (hours). Y represents concentration of cytokine (pg/ml).

7.3 Effect of CsA and bacterial supernatants on reconstructed human Buccal epithelial cultures (RHOE):

7.3.1 Tissue viability:

CsA alone had no significant effect on the viability of RHOE compared to the control (CsA; 119 ± 10 vs. control; 100 ± 15%; Fig 7.5).

P.g, P.i, & F.n significantly increased the cell viability compared to the control (126 ± 2.4%; 128 ± 14%; & 133 ± 2.8% respectively; $p<0.05$ vs. control), whilst addition of CsA had no further effect on viability.

A.a supernatant either individually or in combination with CsA had no significant effect on viability of the RHOE.

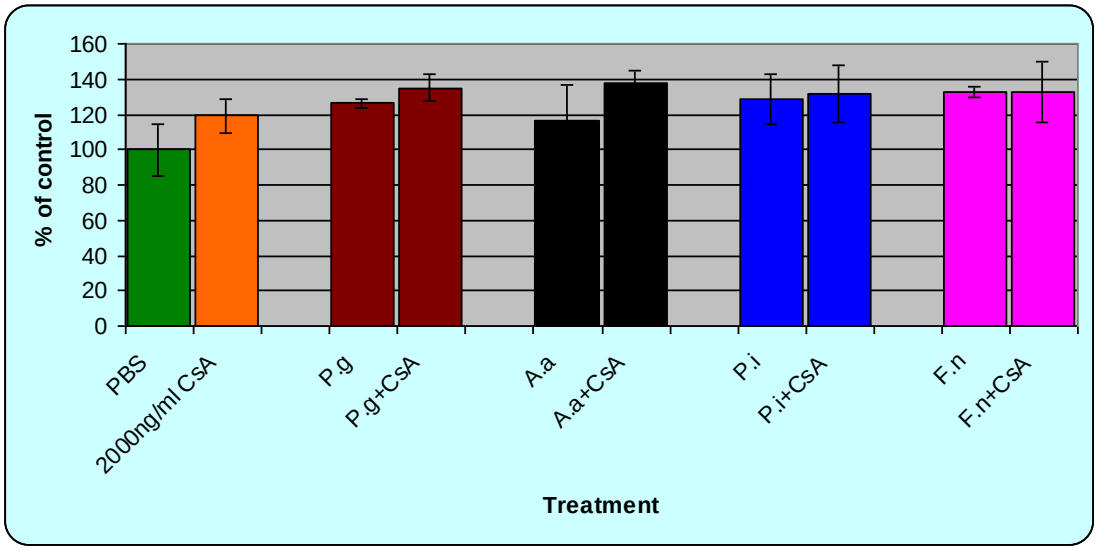


Figure 7.5. Effect of CsA and bacterial supernatants on RHOE viability.
RHOE cultures were stimulated with 2000ng/ml CsA or/and bacterial supernatant. Cell viability was measured by MTT assay after 48h exposure. Control cultures were exposed to PBS. Results are expressed as the mean ± SEM (n=3). Statistical significance was regarded when $p < 0.05$ compared to control.

7.3.2 IL-1α release:

CsA alone had no significant effect on IL-1α release of compared to the control up to 48h (Fig 7.6)

P.g, A.a. P.i, and F.n alone or in combination with CsA had little or no significant effect on IL-1α release up to 48h (Fig 7.6).

7.3.3 IL-6 release:

CsA alone had no significant effect on IL-6 release compared to the control up to 48 h (Fig 7.7).

P.g, A.a, P.i, and F.n alone or in combination with CsA had no significant effect on IL-6 release up to 48h (Fig 7.7).

7.3.4 IL-8 release:

CsA caused a slight but significant reduction in IL-8 release compared to the control (100 ± 12pg/ml 144 ± 18pg/ml at 48h; respectively; $p<0.05$ Fig 7.8).

P.g, A.a, P.i and Fn significantly increased IL-8 release at all time points studied (e.g. 400 ± 43, 436 ± 61, 392 ± 49 & 400± 30 pg/ml at 48h respectively; $p<0.05$ Fig 7.8). CsA had little or no effect at 6 h and 24h. However after 48h, CsA significantly reduced P.g, A.a and P.i induced release of IL-8 (217 ± 30, 272 ± 32, &; 275 ± 57pg/ml; respectively $p<0.05$), although these levels were still significantly greater than controls ($p <0.05$).

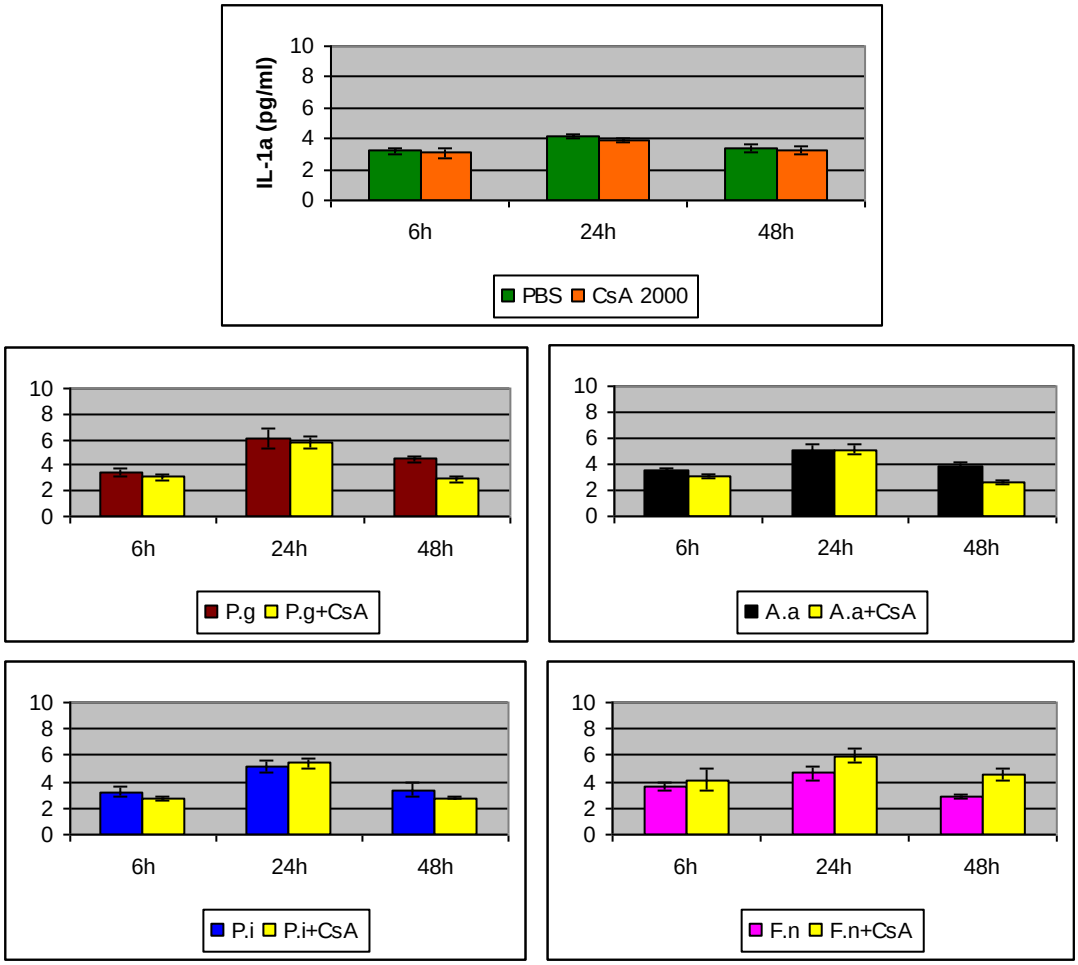


Figure 7.6. Effect of CsA/CsA+bacterial supernatant combinations on release of IL-1α in RHOE. RHOE cultures were stimulated with 2000ng/ml CsA and/or bacterial supernatant. Control cultures were exposed to PBS. IL-1α was measured by Elisa assay after 6h, 24h, and 48h exposure. Results are expressed as the mean ± SEM (n=5). Statistical significance was regarded when $p < 0.05$ compared to control. X axis represents treatment time (hours). Y represents concentration of cytokine (pg/ml).

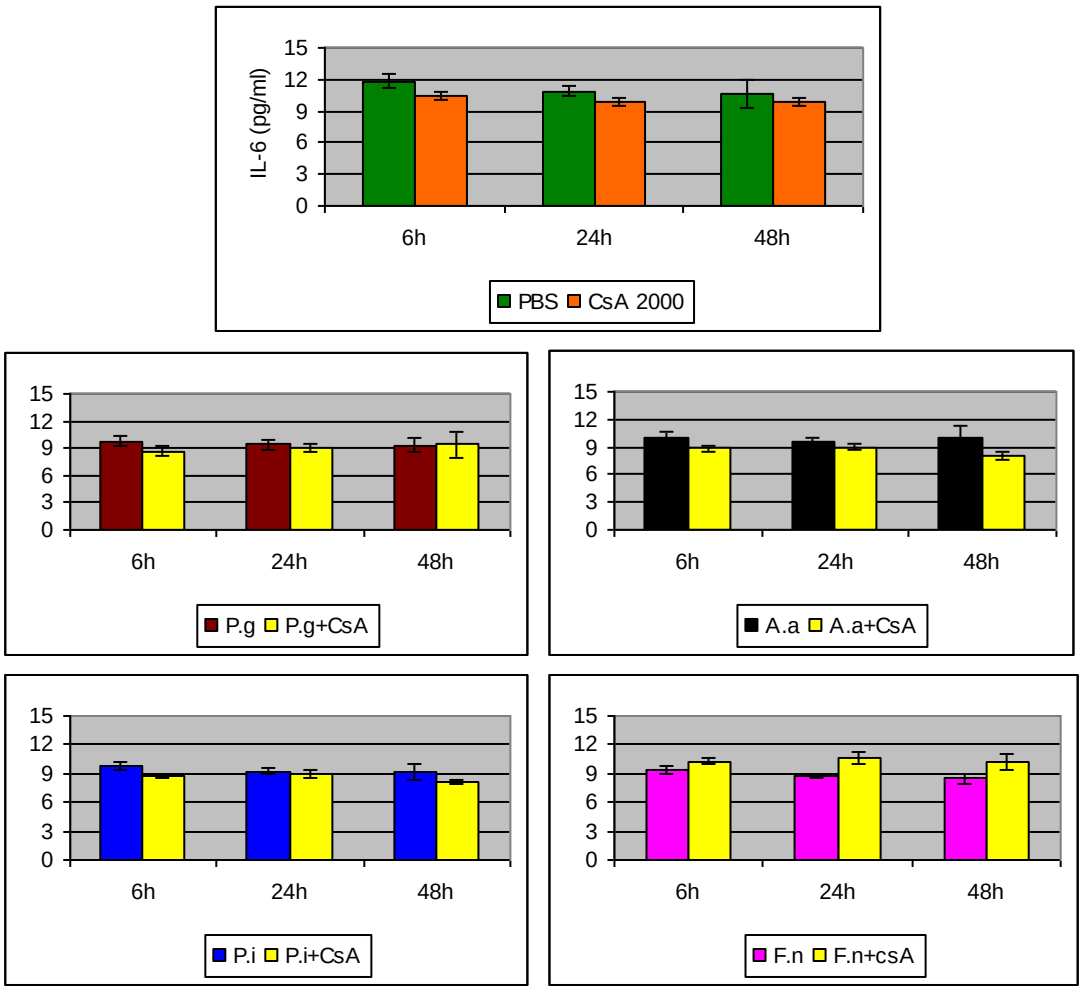


Figure 7.7. Effect of CsA/CsA+bacterial supernatant combinations on release of IL-6 in RHOE. RHOE cultures were stimulated with 2000ng/ml CsA and/or bacterial supernatant. Control cultures were exposed to PBS. IL-6 was measured by Elisa assay after 6h, 24h, and 48h exposure. Results are expressed as the mean ± SEM (n=5). Statistical significance was regarded when $p < 0.05$ compared to control. X axis represents treatment time (hours). Y represents concentration of cytokine (pg/ml).

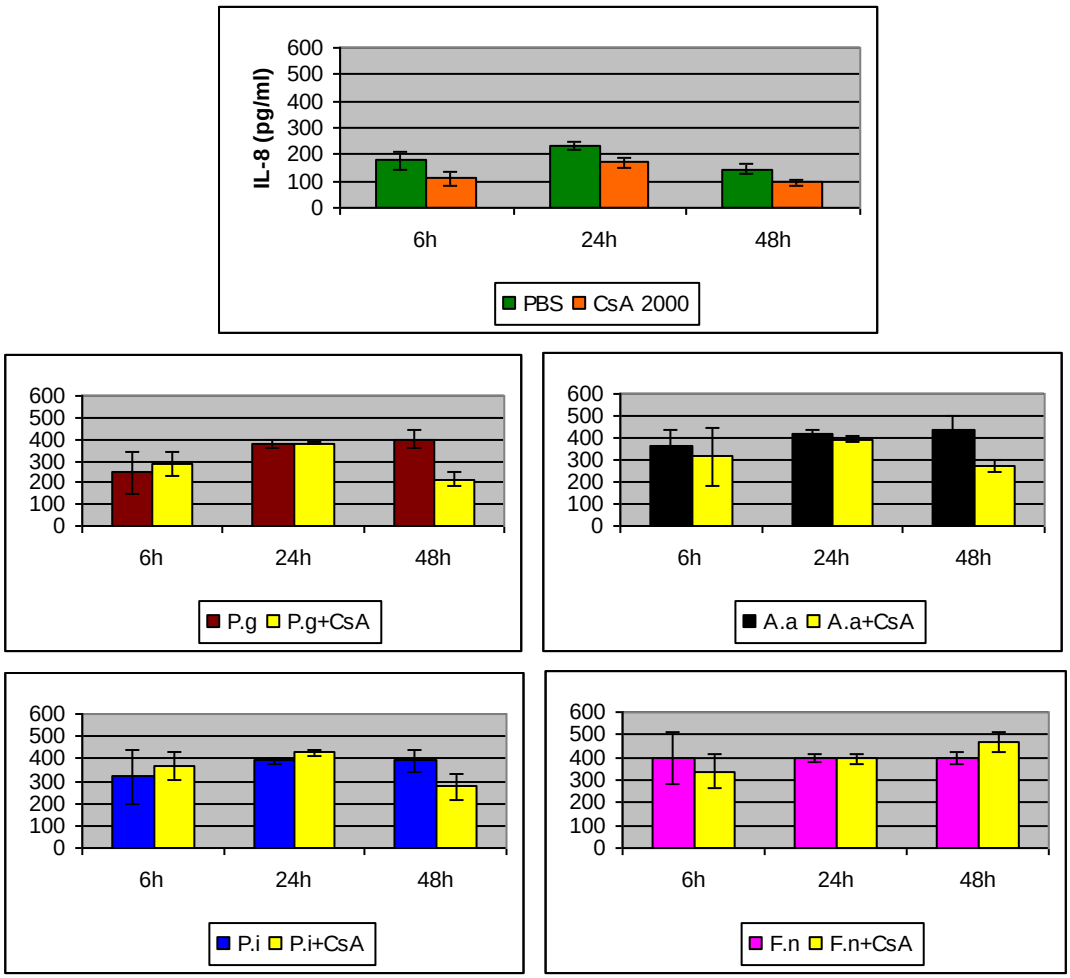


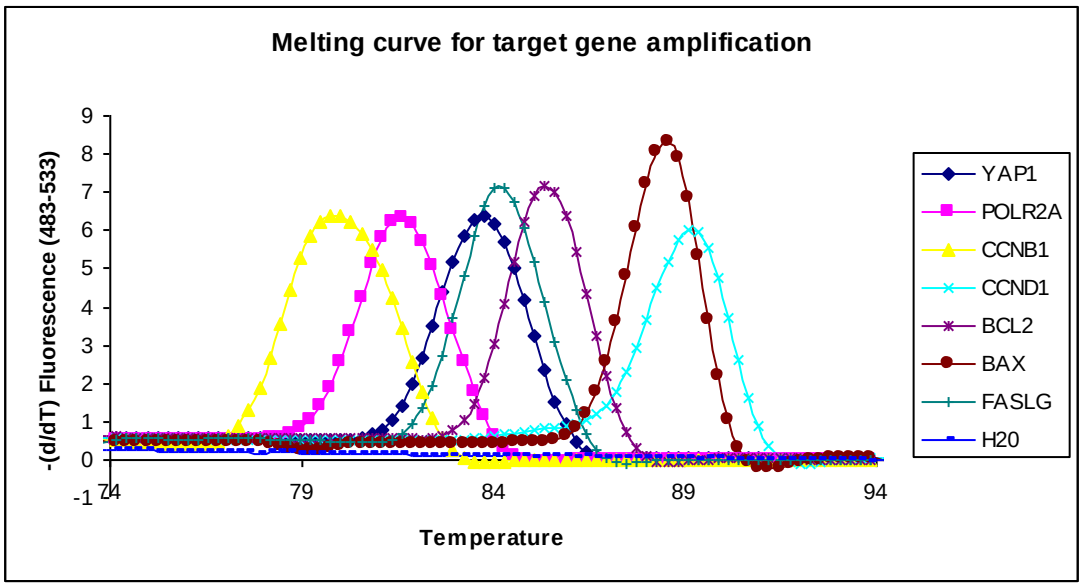
Figure 7.8. Effect of CsA/CsA+bacterial supernatant combinations on release of IL-8 in RHOE. RHOE cultures were stimulated with 2000ng/ml CsA and/or bacterial supernatant. Control cultures were exposed to PBS. IL-8 was measured by Elisa assay after 6h, 24h, and 48h exposure. Results are expressed as the mean ± SEM (n=5). Statistical significance was regarded when $p < 0.05$ compared to control. X axis represents treatment time (hours). Y represents concentration of cytokine (pg/ml).

7.4. Gene expression (qPCR):

7.4.1. Confirmation of primer specificity:

Melting curve analyses revealed that each qPCR amplification generated a single peak at a point corresponding to the product specific melting temperature (Fig 7.9). No primer-dimers were however detected during the 45 amplification cycles in all qPCR reactions. These findings were in agreement with agarose gel electrophoresis that showed a single band for each product corresponding to the predicted length. These results confirmed the absence of non-specific PCR products and thus, the specificity of the primer sets used in this study.

A



B-

Figure 7.9. Confirmation of PCR product specificity. Panel A: melting curve analyses of two house keeping gene and five target gene amplification reactions displays a single melting peak for each PCR product at distinct melting temperature ^{o}C. Water used as control is appearing flat and no primer-dimers were detected during the 45 amplification cycles in all qPCR reactions.
Panel B: the specificity of the same PCR reactions was confirmed by agarose gel electrophoresis that showed single amplification products with the predicted length.
Specificity of the amplification products from the house keeping genes were already confirmed by our staff in the department.

7.4.2. Effect of CsA and bacterial supernatants on gene expression from RHGE:

7.4.2.1 CCNB1 expression:

CsA significantly reduced the expression of CCNB1 compared to control ($p<0.05$; Fig 7.10; Table 7.1). Stimulation with bacterial supernatants alone either significantly decreased (P.g, A.a, and F.n) or had no effect (P.i) on CCNB1 levels. The addition of CsA to RHGE pre-exposed to any of the bacterial supernatants, however, caused a significant increase in CCNB1 levels ($p<0.05$). This effect was most pronounced with P.i, A.a, and F.n ($p<0.05$; Fig 7.10; Table 7.1).

Culture type	Doses applied	CCNB1 mRNA (Mean ± SEM)	Sample (N)
RHGE	Control (PBS)	1 ± 0.047	3
	2000ng/ml CsA	0.70 ± 0.032	3
	P.g	0.70 ± 0.057	3
	P.g+CsA	1.10 ± 0.103	3
	A.a	0.66 ± 0.02	3
	A.a + CsA	1.54 ± 0.039	3
	P.i	1.04 ± 0.056	3
	P.i + CsA	1.41 ± 0.033	3
	F.n	0.69 ± 0.051	3
	F.n + CsA	1.28 ± 0.037	3

Table 7.1. Expression of CCNB1 in RHGE cultures stimulated with PBS, or CsA, or bacterial supernatants, or combination of CsA and bacterial supernatant.
Results were normalized to the control (PBS) and expressed as a fold mean ± SEM.

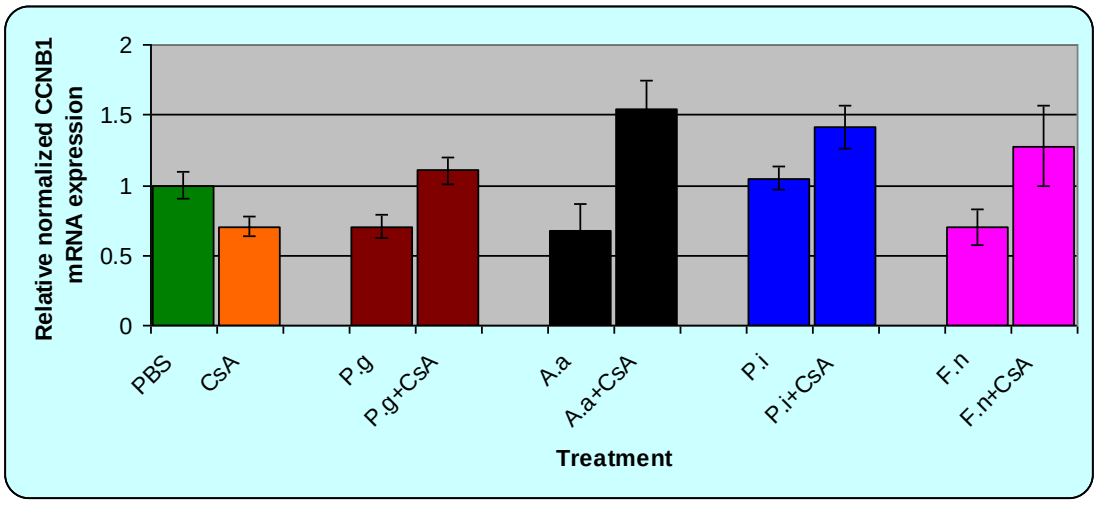


Figure 7.10. Expression of CCNB1 in RHGE exposed to either PBS, or CsA, or bacterial supernatant only, or a combination of CsA and bacterial supernatant.
Results were normalized to both house keeping genes and afterwards to the biologic control PBS. Each bar represents a mean ± SEM of n=3. The significant effect was considered when $p<0.05$.

7.4.2.2 CCND1 expression:

CsA had no effect on CCND1 compared to the control (Fig 7.11; Table 7.2). All the bacterial supernatants however, significantly increased CCND1 expression ($p<0.05$; Fig 7.11 and Table 7.2). CsA, significantly promoted this stimulatory effect on CCND1 mRNA expression.

Culture type	Doses applied	CCND1mRNA (Mean ± SEM)	Sample (N)
RHGE	Control (PBS)	1± 0.106	3
	2000ng/ml CsA	1.046 ± 0.079	3
	P.g	2.03 ± 0.09	3
	P.g+CsA	2.99 ± 0.108	3
	A.a	2.12 ± 0.22	3
	A.a + CsA	3.41 ± 0.22	3
	P.i	1.45 ± 0.09	3
	P.i + CsA	2.77 ± 0.17	3
	F.n	1.73 ± 0.15	3
	F.n + CsA	2.91 ± 0.32	3

Table 7.2 Expression of CCND1 in RHGE cultures stimulated with PBS, or CsA, or bacterial supernatants, or combination of CsA and bacterial supernatant.
Results were normalized to the control (PBS) and expressed as a fold mean ± SEM.

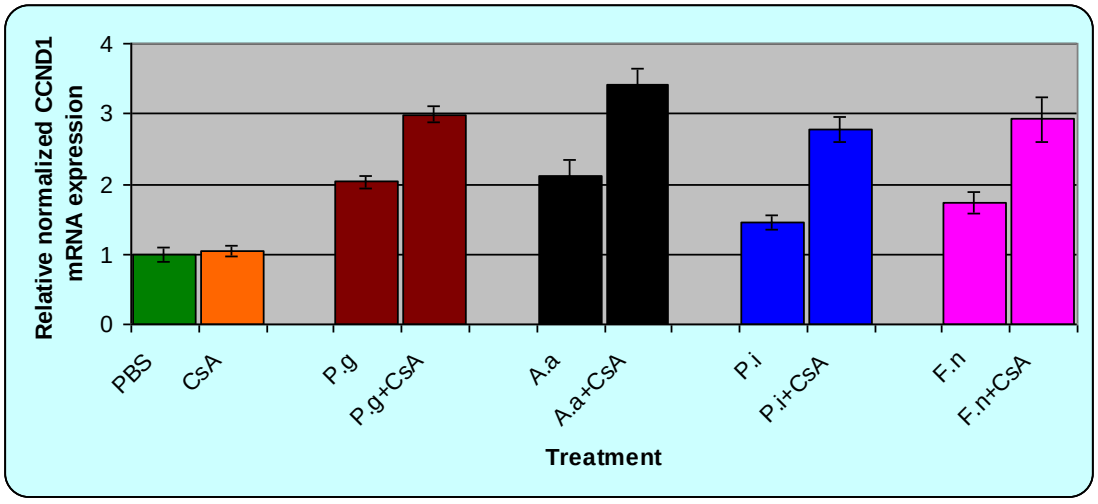


Figure 7.11. Expression of CCND1 in RHGE exposed to either PBS, or CsA, or bacterial supernatant only, or a combination of CsA and bacterial supernatants.
Results were normalized to both house keeping genes and afterwards to the biologic control PBS. Each bar represents a mean ± SEM of n=3. The significant effect was considered when $p<0.05$.

7.4.2.3. Fas-L expression:

CsA and bacteria supernatants had no effect on the expression of Fas-L mRNA in RHGE (Fig 7.12; Table 7.3).

Culture type	Doses applied	Fas-L mRNA (Mean ± SEM)	Sample (N)
RHGE	Control (PBS)	1 ± 0.90	3
	2000ng/ml CsA	1.09 ± 0.58	3
	P.g	8.89 ± 9.09	3
	P.g+CsA	3.54 ± 1.06	3
	A.a	3.14 ± 1.97	3
	A.a + CsA	9.56 ± 9.55	3
	P.i	2.29 ± 0.54	3
	P.i + CsA	1.77± 0.68	3
	F.n	0.74 ± 0.49	3
	F.n + CsA	2.84 ± 0.99	3

Table 7.3 Expression of Fas-L in RHGE cultures stimulated with PBS, or CsA, or bacterial supernatants, or combination of CsA and bacterial supernatant.
Results were normalized to the control (PBS) and expressed as a fold mean ± SEM.

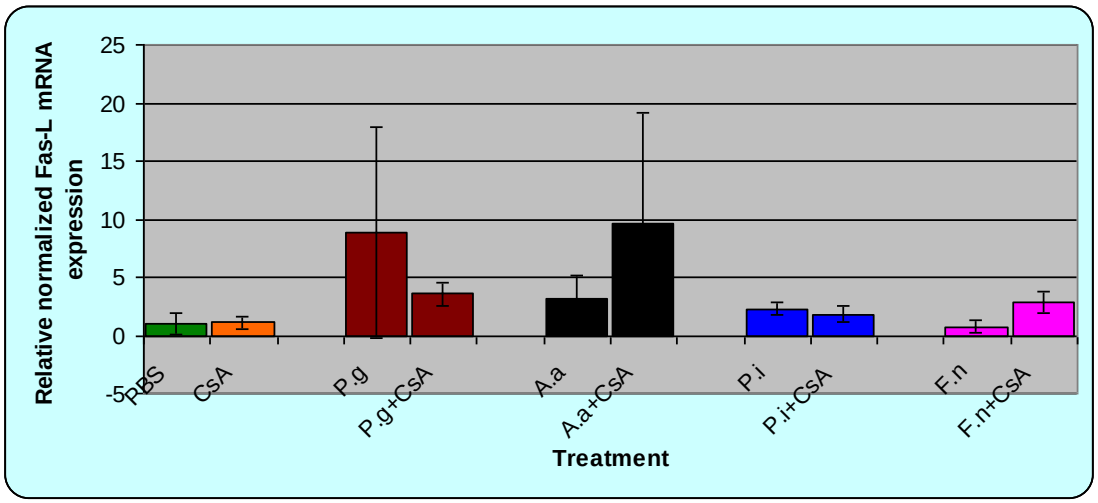


Figure 7.12. Expression of Fas-L in RHGE exposed to either PBS, or CsA, or bacterial supernatant only, or a combination of CsA and bacterial supernatant.
Results were normalized to both house keeping genes and afterwards to the biologic control PBS. Each bar represents a mean ± SEM of n=3. The significant effect was considered when $p<0.05$.

7.4.2.4. Bcl-2/Bax mRNA ratio expression:

CsA and bacteria supernatants had no effect on the expression of Bcl-2/Bax mRNA in RHGE (Fig 7.13; Table 7.4).

Culture type	Doses applied	Bcl2/Bax mRNA (Mean ± SEM)	Sample (N)
RHGE	Control (PBS)	1 ± 0.5	3
	2000ng/ml CsA	1 ± 0.8	3
	P.g	1.6 ± 0.7	3
	P.g+CsA	1 ± 0.6	3
	A.a	1.3 ± 1.1	3
	A.a + CsA	1.1 ± 0.7	3
	P.i	0.7 ± 0.4	3
	P.i + CsA	1.3 ± 0.6	3
	F.n	1.1 ± 0.7	3
	F.n + CsA	0.8 ± 0.5	3

Table 7.4. Expression of Bcl2/Bax mRNA ratio in RHGE cultures stimulated with PBS, or CsA, or bacterial supernatants, or combination of CsA and bacterial supernatant. Results were normalized to the control (PBS) and expressed as a fold mean ± SEM.

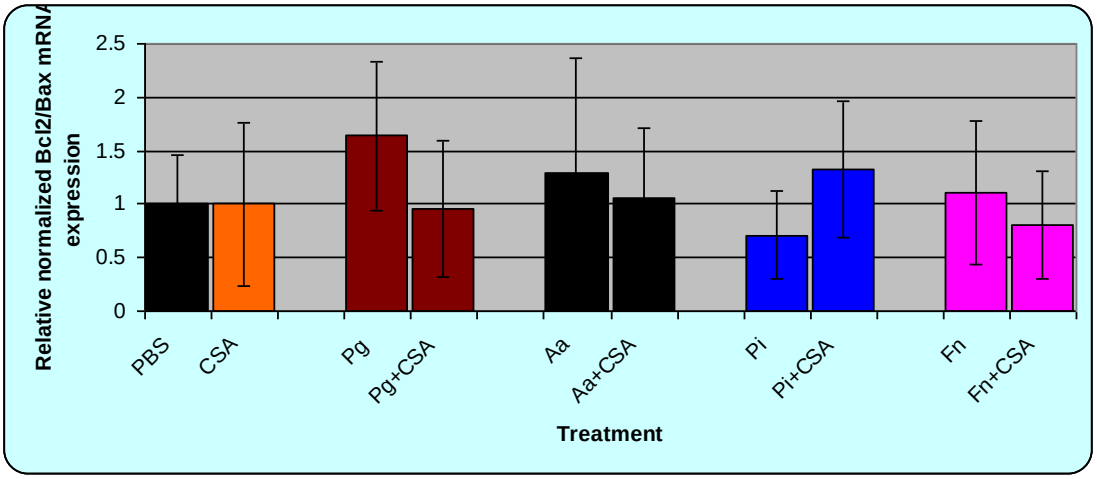


Figure7.13. Bcl-2/Bax mRNA ratio expression in RHGE exposed to either PBS, or CsA, or bacterial supernatant only, or a combination of CsA and bacterial supernatant. Results were normalized to both house keeping genes and afterwards to the biologic control PBS. Each bar represents a mean ± SEM of n=3. The significant effect was considered when $p<0.05$.

7.4.3. Effect of CsA and bacterial supernatants on gene expression from RHOE:

7.4.3.1. CCNB1 expression:

CsA alone had no significant effect on CCNB1 expression (Fig 7.14; Table7.5).
P.g, A.a and P.i alone had no significant effect on CCNB1 expression compared to both control and cultures treated with CsA ($p>0.05$; Fig 7.14; Table7.5) whilst CsA significantly reduced CCNB1 expression compared to that of cultures exposed to the respective bacterial supernatant, cultures treated with CsA, and control culture ($p<0.05$; Fig 7.14; Table7.5).

F.n alone however, significantly increased CCNB1 expression whilst the addition of CsA caused a significant decrease in CCNB1 mRNA levels ($p<0.05$; Fig 7.14; Table7.5).

Culture type	**Doses applied**	**CCNB1 mRNA** (Mean ± SEM)	**Sample (N)**
RHOE	Control (PBS)	1 ± 0.10	3
	2000ng/ml CsA	0.79 ± 0.051	3
	P.g	0.99 ± 0.22	3
	P.g+CsA	0.50 ± 0.017	3
	A.a	1.026 ± 0.22	3
	A.a + CsA	0.36 ±0.019	3
	P.i	0.89 ± 0.098	3
	P.i + CsA	0.48 ± 0.012	3
	F.n	1.44 ± 0.16	3
	F.n + CsA	0.94 ± 0.07	3

Table 7.5 Expression of CCNB1 in RHOE cultures stimulated with PBS, or CsA, or bacterial supernatants, or combination of CsA and bacterial supernatant.
Results were normalized to the control (PBS) and expressed as a fold mean ± SEM.

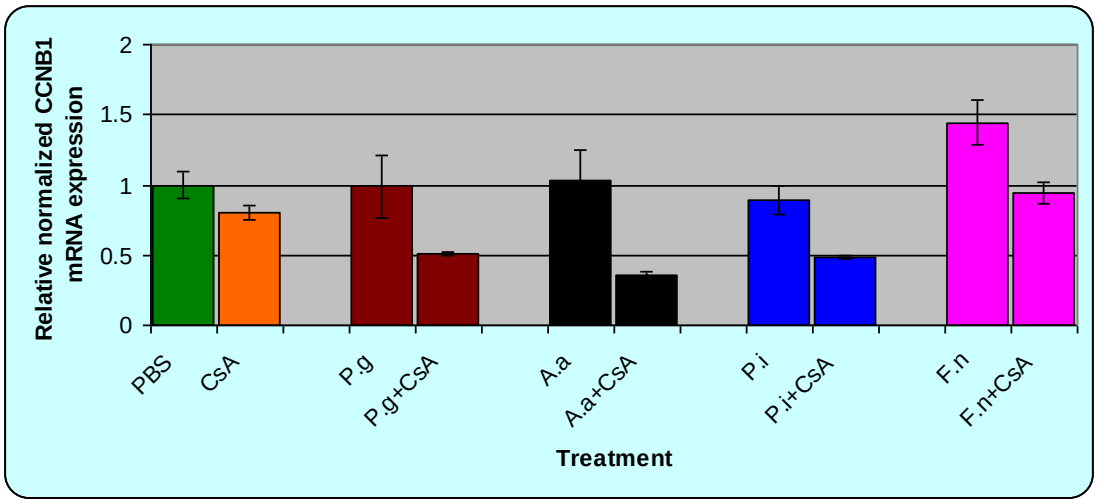


Figure 7.14. Expression of CCNB1 in RHOE exposed to either PBS, or CsA, or bacterial supernatant only, or a combination of CsA and bacterial supernatant.
Results were normalized to both house keeping genes and afterwards to the biologic control PBS. Each bar represents a mean ± SEM of n=3. The significant effect was considered when $p<0.05$.

7.4.3.2 CCND1 expression:

CsA alone, Pg, Pi and F.n had no effect on CCND1 expression compared to the control ($p<0.05$; Fig 7.15; Table 7.6). A.a however caused a significant increase in CCND1 gene expression compared to both control and cultures treated with CsA ($p>0.05$; Fig 7.15; Table 7.6).

Culture type	**Doses applied**	**CCND1mRNA** (Mean ± SEM)	**Sample (N)**
RHOE	Control (PBS)	1± 0.15	3
	2000ng/ml CsA	0.84 ± 0.081	3
	P.g	1.38 ± 0.02	3
	P.g+CsA	1.28 ± 0.040	3
	A.a	1.511 ± 0.108	3
	A.a + CsA	1.65 ± 0.123	3
	P.i	1.33 ± 0.097	3
	P.i + CsA	1.11 ± 0.029	3
	F.n	0.94 ± 0.06	3
	F.n + CsA	1.15 ± 0.10	3

Table 7.6 Expression of CCND1 in RHOE cultures stimulated with PBS, or CsA, or bacterial supernatants, or combination of CsA and bacterial supernatant.
Results were normalized to the control (PBS) and expressed as a fold mean ± SEM.

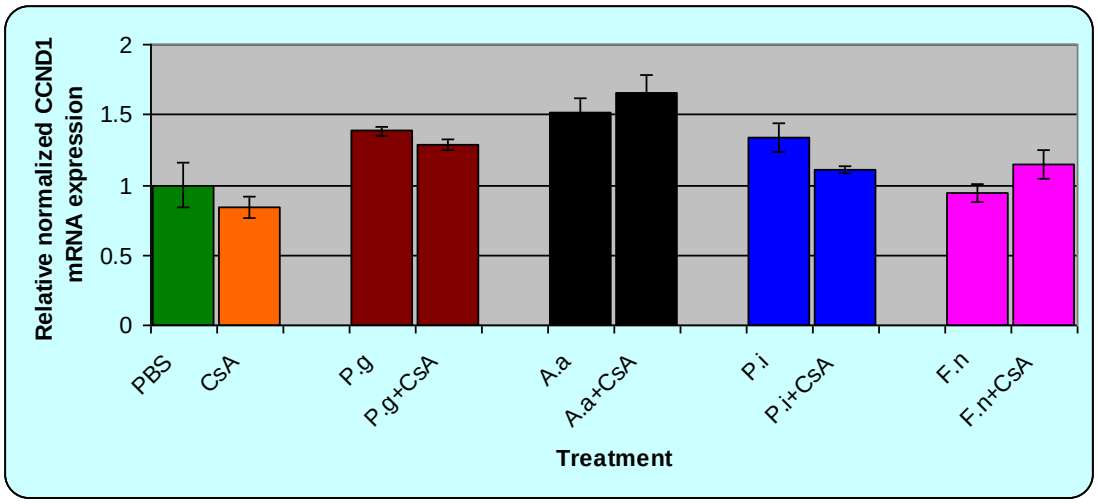


Figure7.15. Expression of CCND1 in RHOE exposed to either PBS, or CsA, or bacterial supernatant only, or a combination of CsA and bacterial supernatant. Results were normalized to both house keeping genes and afterwards to the biologic control PBS. Each bar represents a mean ± SEM of n=3. The significant effect was considered when $p<0.05$.

7.4.3.3. Fas-L mRNA expression:

CsA and bacteria supernatants had no effect on the expression of Fas-L mRNA in RHOE (Fig 7.16; Table 7.7).

Culture type	Doses applied	Fas-L mRNA (Mean ± SEM)	Sample (N)
RHOE	Control (PBS)	1± 0.64	3
	2000ng/ml CsA	0.93 ± 0.819	3
	P.g	4.05 ± 4.08	3
	P.g+CsA	2.80 ± 1.277	3
	A.a	1.25 ± 0.34	3
	A.a + CsA	3.22 ± 3.76	3
	P.i	1.23 ± 1.16	3
	P.i + CsA	0.617± 0.51	3
	F.n	2.26 ± 2.74	3
	F.n + CsA	2.16 ± 2.65	3

Table 7.7. Expression of Fas-L mRNA in RHOE cultures stimulated with PBS, or CsA, or bacterial supernatants, or combination of CsA and bacterial supernatant. Results were normalized to the control (PBS) and expressed as a fold mean ± SEM.

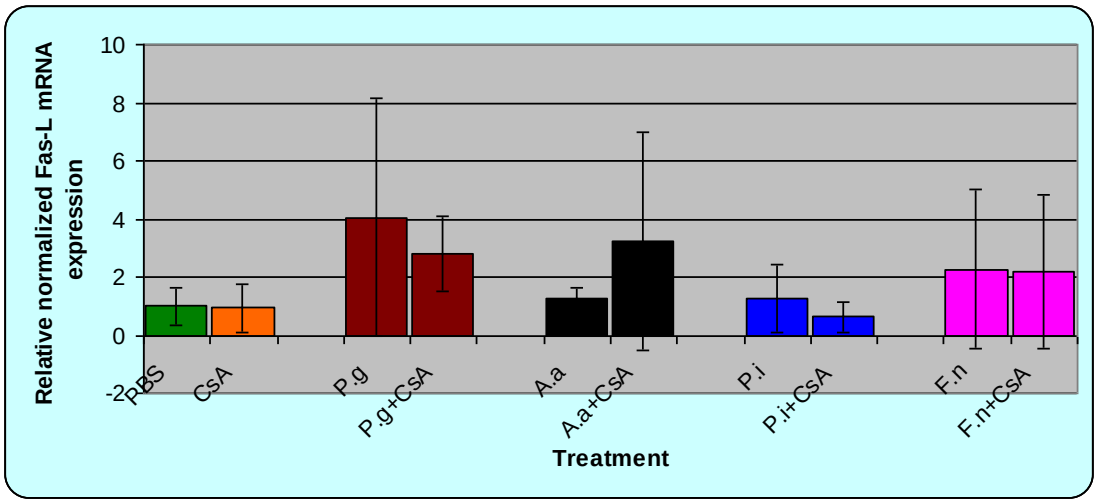


Figure7.16. Expression of Fas-L mRNA in RHOE exposed to either PBS, or CsA, or bacterial supernatant only, or a combination of CsA and bacterial supernatant. Results were normalized to both house keeping genes and afterwards to the biologic control PBS. Each bar represents a mean ± SEM of n=3. The significant effect was considered when $p<0.05$.

7.4.3.4. Bcl-2/Bax mRNA ratio expression:

CsA and bacteria supernatants had no effect on the expression of Bcl-2/Bax mRNA in RHGE (Fig 7.17; Table 7.8).

Culture type	Doses applied	Bcl2/Bax mRNA (Mean ± SEM)	Sample (N)
RHOE	Control (PBS)	1 ± 0.2	3
	2000ng/ml CsA	1.1 ± 0.2	3
	P.g	1.2 ± 0.1	3
	P.g+CsA	0.9 ± 0.2	3
	A.a	2.1 ± 1.2	3
	A.a + CsA	1.6 ± 0.6	3
	P.i	1.1 ± 0.1	3
	P.i + CsA	1.5 ± 1.1	3
	F.n	1.2 ± 0.6	3
	F.n + CsA	1.5 ± 0.7	3

Table. 7.8 Expression of Bcl2/Bax mRNA ratio in RHOE cultures stimulated with PBS, or CsA, or bacterial supernatants, or combination of CsA and bacterial supernatant. Results were normalized to the control (PBS) and expressed as a fold mean ± SEM.

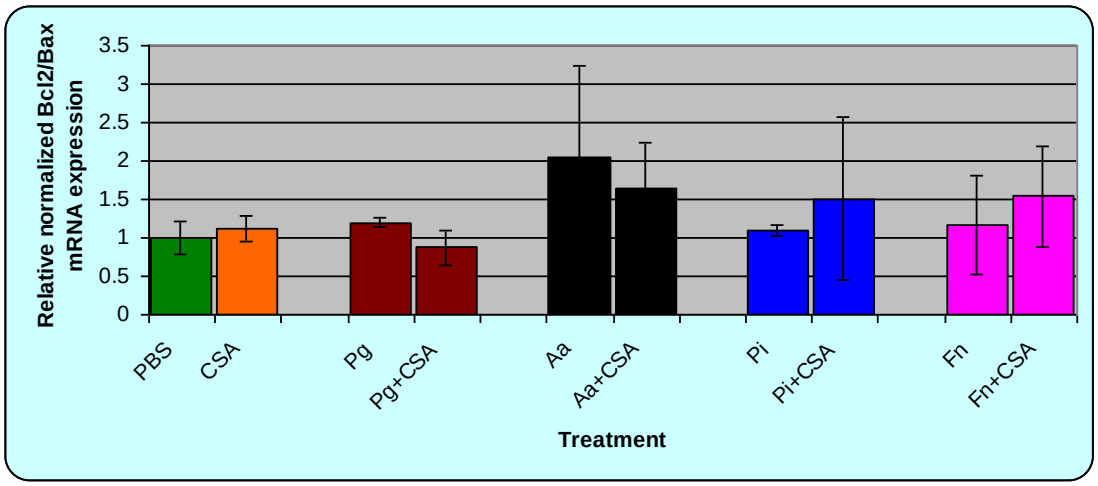


Figure7.17. Bcl-2/Bax mRNA ratio expression in RHOE exposed to either PBS, or CsA, or bacterial supernatant only, or a combination of CsA and bacterial supernatant. Results were normalized to both house keeping genes and afterwards to the biologic control PBS. Each bar represents a mean ± SEM of n=3. The significant effect was considered when $p<0.05$.

7.5 Summary:

CsA alone had no effect on RHGE viability, whilst P.g, A.a, and P.i supernatants caused a significant reduction. In contrast a combination of CsA plus any of the bacterial supernatants increased the viability with the most pronounced effect being found with CsA/F.n where the level of viability was significantly higher than control. None of the treatments applied had a significant effect on the release of IL-1α and IL-6 from RHGE. CsA, P.g, F.n individually had no effect on IL-8 release whereas A.a and P.i caused a a significant increase after 24h. However, addition of CsA reduced the level with values returning to the control level.

CsA alone, P.g, A.a, and F.n caused a significant reduction in CCNB1 expression in RHGE. Interestingly, exposure to combinations of CsA and any of bacterial supernatant reversed that effect to a stimulatory effect. All bacterial supernatants significantly up-regulated the expression of CCND1 but no effect was found with CsA alone. Furthermore, exposure to combinations of CsA and any of bacterial supernatants significantly promoted this stimulatory effect on CCND1 expression.

In contrast to RHGE, P.g, P.i, and F.n caused a significant increase in viability of RHOE but no effect was found with CsA alone and A.a. Furthermore, all combinations had no significant effect on the viability of RHOE. Similarly to RHGE, all treatments had no effect on the release of IL-1α and IL-6. However, CsA alone reduced the release of IL-8 whilst all bacterial supernatants caused a significant increase in release of this cytokine at all time intervals.

CsA alone, P.g, A.a, and P.i had no significant effect on CCNB1 expression but surprisingly, all combination of CsA and bacterial supernatants significantly down-regulated its expression. However, expression of CCND1 was not significantly modulated under influence all of the treatments applied.

Expression of apoptosis-related genes has not been modulated under influence any of the treatments applied in both RHGE and RHOE cultures.

Collectively, CsA alone had a little effect on both RHGE and RHOE. However, these types of epithelium reacted differently when exposed to combinations of CsA and bacterial supernatants. All combinations stimulated the proliferative activity of RHGE by reversing the inhibitory effect of CsA and bacterial supernatants alone on CCNB1 expression and promoting the stimulatory effect of bacterial supernatants on CCND1 expression, In contrast these combinations of CsA and bacterial supernatants inhibited the proliferative activity of RHOE by down-regulating the expression of CCNB1, but had no effect on CCND1.

Chapter Eight
Discussion

The mechanism underlying CsA-induced gingival hyperplasia is unclear (Cotrim *et al.,* 2003; Yoshida *et al.,* 2005; Birraux *et al.,* 2006) and therefore, the main objective of our study was to evaluate the direct effect of CsA and to investigate whether the exposure to a combination of CsA and bacterial products might modulate the response of gingival and oral cells *in vitro*. This was evaluated using monolayer cultures of three keratinocyte cell lines (OKF6, FIBS and TR146) and HGF derived from human gingival fibroblasts and a stratified model oral and gingival epithelium (SkinEthic, Nice).

8.1 Monolayer cultures:

8.1.1 Cell viability:

CsA alone exhibited a variable effect on the viability of the different cell lines. FIBS, OKF6 and HGF showed an increase whilst there was no change in TR146. The concentrations chosen reflected physiological and local gingival tissue levels (Fisher *et al.,* 1988; McGaw *et al.,* 1987) and the peak effect was, in general present within 24hrs. In contrast to previous studies (Barber *et al.,* 1992), prolonged exposure had no effect or reduced viability rather than leading to an increase.

Pre-stimulation of cells with bacterial supernatants also showed a variable effect on cell viability. Keratinocyte viability was increased by F.n and P.i, whilst P.g caused an increase in OKF6 and TR146 but a decrease in FIBS and A.a caused a decrease in OKF6 and an increase in TR146. HGF showed no change (P.g, P.i) or inhibition with A.a and F.n.

The MTT assay, however, only provides limited information about the modulation in cell viability and is unable to determine whether the cells are able to replicate and hence does not differentiate between cycling cells or static cells. CsA has been reported to alter cell proliferation without change in cell viability (Willershausen-zönnchen *et al.,* 1992) and therefore more sophisticated assays were required to evaluate the cellular response to CsA and bacterial products and whether this response involved modulation in the local inflammatory response.

8.1.2 Cell proliferation:

To gain an insight into the proliferative activity a flow cytometric assay was developed to simultaneously determine changes in cell cycle distribution, rate of DNA synthesis as determined by Edu incorporation, and expression of Cyclins D1 and B1. CsA, using this approach had a variable effect on the cell cycle distribution, Edu incorporation and Cyclin D1 expression. These results are in keeping with previous studies in which CsA caused an inhibition in proliferation of human endothelial and renal tubular epithelial cells but not human skin fibroblasts (Esposito *et al.,* 2000) and inhibited melanocyte proliferation in a dose-dependent manner with no apparent effect on cell viability (Lee & Kang, 2003). CsA has also been reported to inhibit DNA synthesis and proliferation of normal and transformed keratinocyte cell lines (Furue *et al.,* 1988). Furthermore, Karashima *et al* (1996) showed that CsA inhibited human epidermal keratinocytes proliferation by arresting cells at the G0/G1 phases of cell cycle. Blocking of cell cycle in G1 phase has been suggested to be a potential mechanism of CsA-induced proliferation inhibition of cultured human epidermal keratinocytes (Khandke *et al.,* 1991). Kato *et al* (1987) found that CsA within the therapeutic levels did not inhibit the proliferation of animal epidermal cells. Collectively, these findings indicates that the sensitivity of cells to CsA is varied which could explain the different responses of various types of keratinocytes to CsA.

Previous attempts to evaluate the direct effects of CsA on proliferation of HGF resulted in conflicting data ranging from an increase (Cotrim *et al.,* 2003), decrease (Pistorius *et al.,* 2003) or no effect (James *et al.,* 1995). These discrepancies may stem from differences in the methodologies and experimental conditions dose, duration (Kataoka *et al.,* 2000). Furthermore, the variability in the response to CsA among HGF strains has previously been reported (Tipton *et al.,* 1991). These findings were supported by other reports revealing that HGFs derived from nifedipine-responder patients showed higher proliferative rate, illustrated by enhanced BrdU incorporation and upregulated mRNA expression of Cyclin B1 and D1 as well as more promoted cell cycle progression than those derived from nifedipine-non responder individuals (Takeuchi, 2004; Takeuchi *et al.,* 2007). Thus, the heterogeneity among the HGF populations may also account for the different responses to CsA between HGF derived from patients with CsA-induced gingival hyperplasia and those derived from healthy or CsA-non responder individuals (Sukkar *et al.,* 2007).

Recent attention has been drawn to the gingival keratinocytes as a potential part of the pathogenesis of CsA-associated gingival hyperplasia but findings are still inconclusive (Cetinkaya *et al.,* 2006). Yoshida *et al* (2005) assessed proliferation of cultured rat gingival cells after incubation for 3days using MTS assay showing that CsA at concentration of 200-800ng/ml induced hyper proliferation of gingival cells by 23-25% compared to control. However, in this study they used a mixture of keratinocyte and mesenchymal cells. In contrast, Birraux *et al* (2006) demonstrated that low concentration of CsA had no effect on the proliferation of an immortalized normal human oral keratinocyte cell line (HOK-16B), primary oral keratinocytes, and epitheloid cervical carcinoma cell line (Hela) cultured for up to 96h. They did report, however, that higher concentration 10^3ng/ml of CsA exhibited an inhibitory effect on proliferation of all cell types. Furthermore (Lauer *et al.,* 2006) showed both the inhibitory and stimulatory effect of CsA on human gingival keratinocyte proliferation; CsA significantly reduced their proliferation rate from day 3 to day 6 and then stimulated their proliferation from day 6 to day 9 with maximal increase being observed on day 9 at concentration 10^3ng/ml. It is possible that these different findings may be related to different methodologies used in each study.

The exposure to combinations of bacterial supernatants with CsA also caused a variable cell type-dependent effect. The proliferation rate (Edu incorporation) was increased in FIBS, OKF6, and HGF cultures exposed to P.i and 2000ng/ml CsA. These results were supported by an earlier study conducted by Bartold (1989) who found that CsA up-regulated DNA synthesis and enhanced proliferation rate of HGF after treatment with CsA with the most noticeable stimulation being reported in presence of 10%FBS. This capacity was also retained even in absence of FBS or in the presence of lipopolysaccharide that would normally inhibit proliferation of fibroblasts (Bartold, 1989).

2000ng/ml CsA / F.n, in contrast, caused a reduction in proliferation rate of HGF with a corresponding decrease in G2/M fraction. Barber *et al* (1992) demonstrated that addition of CsA to HGFs pre-exposed to a lipopolysaccharide derived from Fusobacterium nucleatum or Escherichia coli changed the inhibitory effect of these

bacterial products on DNA synthesis to a stimulatory effect instead. However, this discrepancy between our findings and their results may simply be interpreted by the different bacterial products used as we used the whole bacterial products while they used only LPS. Collectively, our findings along with the above mentioned data suggests that the bacterial products, in particular P.i, may be an essential co-factor in the pathogenesis of CsA- associated gingival hyperplasia and may explain, in part, why gingival hyperplasia mostly occurs in areas of intensive dental plaque accumulation (Barber *et al.*, 1992; Bartold, 1989).

8.1.3 Cell cycle:

An apparent blockade of the cell cycle machinery at G2/M was observed in all keratinocyte cell cultures exposed to A.a products. However, such effect was not observed in HGF culture. Instead, an enhanced proliferation rate with corresponding increase in S and G2/M phase was found in HGF cultures exposed to a combination of 2000ng/ml CsA and A.a product. It is possible that this variation may reflect different cell surface receptors used by A.a in each cell type (Kent *et al.,* 1998). However, White *et al* (1998) found that A.a products caused an inhibition in HGF proliferation by blockade of the cell cycle at G2/M phase and down regulating cyclin B1 expression.

In the present study, cell cycle analysis showed that the magnitude of serum starvation influence in arresting cell cycle was cell type dependent. While 80% of OKF6 cells were arrested in G1, a less effective arrest was seen in both FIBS (60%) and HGF (57%).

This deficiency in arresting of all cell line types at G0/G1 following 24h of serum starvation was consistent with previous study that observed that 72h of serum starvation could not synchronize all the mammalian cell lines studied but an effective arrest was seen in human breast cancer cell lines (95%) (Gao & Dou, 2000). Thus these discrepancies in cell type-dependent sensitivity to serum-starvation and therefore in cell synchronization may reflect the differences in expression level of Cyclin D1 between cells. However, the cell cycle analysis did not provide a conclusive profile and it was not accurately corresponding to the findings of

proliferate rate. This discrepancy in the value of S phase determined by both DNA content and Edu incorporation may be interpreted by the in-accuracy of cell cycle staining often resulting in overlapping the phases together (Takeuchi *et al.,* 2007) or because of the discrepancy in the incubation periods cells were pulsed by Edu for 1h while incubated with cell cycle dye for 30 minutes.

Cyclin D1 expression is normally present during G1 Phase (Juan & Cardo, 2001). However in this study cyclin D1 was present in keratinocytes throughout all the cell cycle phases. Such aberrant expression of cyclin D1 was noticed in most cancer cell lines (Collecchi *et al.,* 2000; Juan *et al.,* 1996). Therefore, this type of expression could also be related to the method of transforming our keratinocyte cell lines.

HGF, however, showed a typical pattern of cyclin D1 expression being found in G1 phase. Furthermore, this difference in the pattern of cyclin D1 expression between keratinocyte and fibroblast cell lines could be related to the type of cells and the way of transformation to cell lines.

An increase in the proliferation rate, illustrated by DNA synthesis, with an increase in the viability of HGFs after CsA treatment has also been reported recently (Chae *et al.,* 2006; Kim *et al.,* 2008)

Although Cyclin B1 expression has been reported in fibroblasts (Parkar *et al.,* 2004) it was not expressed by any of the cells in our study. It is not clear why this occurred but it may reflect the instability of the proteins under the fixation conditions applied or low levels of expression in the studied cells.

8.1.4 Pro-inflammatory cytokine release:

It has been suggested that pathogenesis of CsA-induced gingival hyperplasia may involve alteration in the expression levels of various inflammatory cytokines in the gingival tissues (Ruhl *et al.,* 2004) who found an increase in the saliva content of IL-1α, IL-6, and IL-8 in patients with CsA-induced gingival hyperplasia. Williamson *et al* (1994) also demonstrated that CsA-hyperplastic gingiva had a significantly higher expression of the IL-6 gene compared to control gingiva. Theses studies and most of the previous *in vitro* reports (Myrillas *et al.,* 1999; Morton & Bagtzoglou, 1999) have

focused on the potential role of alterations in cytokine release in the pathogenesis of altered functions of gingival fibroblasts in response to CsA. There are, however, no reports available on implication of these cytokines in the epithelial changes induced by CsA.

2000ng/ml CsA consistently caused a significant increase in IL-1α and IL-6 from all keratinocyte cell lines with a maximal release being found in the period between 12h and 24h. These results were consistent with a previous study that reported an increase in IL-1α from cultured human epidermal keratinocytes after CsA treatment (Marionnet *et al.,* 1997) and by an *in vivo* study that showed that stimulation of hair growth by CsA in nude mice was associated with up-regulated expression of IL-1α (Gafter-Gvili *et al.,* 2003). However, this increase in IL-1α release might be attributed to the irritating effect of the high dose of CsA that may cause a perturbation in the permeability of cell membrane and thus leaking the intracellular content of pre-formed IL-1α. This suggestion therefore may explain the lack of effect of low doses of CsA on IL-1α release and the early release of IL-1α induced by high doses of CsA and its depletion afterwards. Moreover, the corresponding release of IL-6 from the same cultures might support the notion of amplifier effect of IL-1α on inflammatory response and thus, suggesting that this pattern of IL-6 release might be IL-1α receptor-dependent (Feliciani *et al.,* 1996; Bonifati & Ameglio, 1999).

2000ng/ml CsA also caused a significant increase in release of IL-6 and IL-8 from HGF cells but with no change in IL-1α. These results are consistent with previous studies revealing a significant increase in IL-6 expression from HGF cultures following treatment with CsA (Chae *et al.,* 2006; Morton & Bagtzoglou, 1999). Myrillas *et al* (1999) also found that expression of IL-1β was undetectable in both normal and overgrown gingival fibroblast cultures exposed to CsA but in contrast to our findings; they reported a dose dependent manner inhibition in IL-6 release in both HGF strains with a maximal inhibition being reported at 2000ng/ml CsA. However, these discrepancies in findings may be attributed to heterogeneity of HGF strains (Bagtzoglou *et al.,* 1998).

Furthermore, Leonardi *et al* (2001) found that the exposure to CsA for 24h caused a significant increase in IL-6 and IL-8 release with a parallel decrease in IL-1β release

along with a reduction of cultured human conjunctival fibroblasts viability. In this study they related their findings to the toxic activity of the high doses used of CsA. However, our results showed no such correlation between cell viability and release of cytokines.

The up-regulated release of IL-8 and IL-6 in an IL-1α-independent manner observed in our HGF cultures treated with CsA has also been reported in a recent study in which it was suggested that IL-6 and IL-8 release in HGFs may be mediated by Toll-like cell surface receptors (TLRs) (Suzuki *et al.,* 2009). This suggestion is in parallel with an earlier report showing that IL-6 and IL-8 release was not caused by the signal transduction induced via IL-1α but rather, occurred independently of IL-1α receptor IL-1R (Kent *et al.,* 1998). Similarly, Cheng *et al* (2008) found that exposure of human cervical carcinoma epithelial cells to Chlamydia trachomatis caused over expression of IL-6 and IL-8 before IL-1α was released suggesting that IL-6 and IL-8 release was independent of cell surface IL-1R receptor signalling pathway.

An increase in IL-6 release was also found in primary cultures of HGFs derived from healthy individual after stimulation with CsA and the magnitude of this release varied among HGF cultures according to the donors (Morton & Bagtzoglou, 1999). However, in their study, CsA appeared to abrogate the stimulatory effect of bacteria on expression of IL-6 in HGFs. Interestingly, Maita *et al* (2002) reported that release of IL-6 in CsA-treated HGFs derived from periodontitis sites was much higher than that in CsA-treated HGFs derived from healthy gingiva but however, CsA failed to induce IL-β and IL-8 release in any of HGF strains used.

Bagtzoglou *et al* (1998) attributed the discrepancies in studies in terms of cytokine release to differences in the cytokine secretory phenotype among HGF populations suggesting that the increased expression of IL-6 and IL-8 by HGF in periodontitis sites may be interpreted by predominance of subpopulations with higher cytokine expression capacity. Considering that cytokines are key mediators of the gingival inflammatory response, the aforementioned suggestion which may be clinically relevant and explain the discrepancies in the susceptibility of individuals to dental plaque may also interpret the variable release of cytokines among HGF strains in

response to CsA. Thus, it may explain at least in part, the variable susceptibility among individuals to develop CsA-associated gingival changes.

Our study showed that bacterial supernatants induced a consistent effect on cytokine release in all cell lines, A.a caused a significant increase in IL- 1α release whilst F.n and P.i reduced the release. A.a, P.i, and F.n caused a significant increase in IL-6 release in most cell lines whilst, IL-8 was increased in HGF by all bacterial supernatants except P.g. Furthermore, bacterial supernatants had limited effect on IL-8 release in keratinocytes. Interestingly, P.g caused a complete inhibition in IL-6 and IL-8 release in all cell lines studied.

Combinations of CsA plus bacterial supernatants had no synergistic effect on IL-1α release. However, effect of the combinations on IL-6 release had bacterial and cell type-dependent manner; in FIBS cells, a synergistic effect was found with combinations of CsA plus P.i/F.n. Likewise, such synergistic effect on release of IL-6 and IL-8 was found in HGF with combinations of CsA and all bacterial supernatants except P.g. However, no synergistic effect on IL-8 release was found in all keratinocytes.

Although P.i, F.n and A.a have been shown to be strong stimulators of IL-8 in human gingival and oral epithelial cells (Han *et al.*, 2000; Sfakianakis *et al.*, 2001), no prominent effect was reported in our study in combination with CsA in all keratinocytes. Furthermore, the differences in the ability of bacteria to stimulate or inhibit cytokine release could be attributed to the unique receptors and signal transduction pathways used by each stimulant (Kent *et al.*, 1998). Therefore, it is possible to speculate that the different receptors and signalling pathways used by CsA and bacterial products in each cell type may cause the different levels of cytokine expression among the cultures.

However, the increase in IL-6 and IL-8 release induced by a synergistic effect of CsA plus bacterial supernatants found in HGF and FIBS (IL-6 only) may be a clinically relevant where this up-regulation in inflammatory cytokines release may enhance the recruitment and activation of inflammatory cells in addition to enhancing the interaction between these inflammatory mediators and gingival cells and ultimately

establishing the inflammatory process in the periodontal tissue that might lead to gingival enlargement. Therefore, this synergistic effect of CsA and bacterial supernatant combinations may provide further explanation of why the CsA-induced gingival hyperplasia is mainly associated with areas of heavy dental plaque accumulation

8.1.5 Apoptosis

Recently, it was reported that the increased number in HGF observed in CsA-induced gingival hyperplasia was the result of reduced apoptosis coupled with increased proliferative activity (Kantarci *et al.,* 2007). Furthermore, Nishikawa *et al.,* (1996) suggested that the excessive number of fibroblasts in drug-induced gingival hyperplasia may be caused by inhibition of apoptosis. Jung *et al.,* (2008) reported that CsA increased the cell viability of HGF cultures *in vitro* and this increase was resulted from a reduced apoptosis, illustrated by reduced expression Bax/Bcl-2 ratio, rather than increased cell proliferation.

Keratinocytes showed that the basal level of Bax expression was ranging from undetectable to a very low level. CsA had no effect on Bax expression in all keratinocytes cultures. Furthermore, a pronounced expression of Bax was found consistently in all keratinocyte cultures exposed to A.a products. However, adding CsA to cultures exposed to A.a caused a cell type-dependent effect; ranging from reduction in OKF6 to an increase in TR146. The result in TR146 is supported by a recent study in which high doses of CsA increased the Bax expression in a human gingival squamous carcinoma cell line (Tu *et al.,* 2009).

CsA induced Bax expression in HGF. Such expression was also observed in all HGF exposed to all bacterial products with more prominent expression was being found in P.i, addition of 2000ng/ml caused an apparent reduction in Bax expression. However, this reversal effect exerted by CsA might explain partly why this pathogenesis mostly seen in areas with heavily dental plaque accumulation and indicate that CsA might acts in different pathways where it up-regulates Bax expression when applied individually but it downregulates this expression when applied in combination with bacterial products.

It was surprising that Bcl-2 expression was not present in any of the cells studied. However, the half-life of theBcl-2 gene and its protein are unstable and differ according to the cell type (Seto *et al.,* 1988; Blagosklonny *et al.,* 1996) cell systems (Gao & Dou, 2000) or in response to various stimuli. In support of this, Buduneli *et al* (2003) reported that the P53 and Bcl-2 contents in gingival crevicular fluid (GCF) of patients with CsA-induced gingival hyperplasia were below the minimum detectable ranges Furthermore, Birraux *et al* (2006) also found that an anti-proliferative effect of CsA on immortalized normal human oral keratinocyte cell lines (HOK-16B) and primary oral keratinocyte cultures but this effect was not associated with changes in the level of TNF-α- induced apoptosis, or Bcl-2 expression. The absence of Bcl-2 expression in our cultures therefore can be explained by instability of this protein under the fixation process followed, or that it is expressed in low levels in these cells, or that the half-life of Bcl-2 in these cells is very short thus its expression may be undetectable.

Despite the limitations of our study including only one sample for each treatment group and being our study focused on two mediators of apoptosis, these findings will lay the foundation for future studies directed towards the importance of periodontal bacteria, in particular P.i, in pathogenesis of CsA-induced gingival hyperplasia.

8.2. Multilayer cultures:

Monolayer cultures lack the multilayered structure of normal mucosa and consequently the concentrations of an agent required to induce responses in these cultures are several orders of magnitude lower that those which cause such response *in vivo* (Coquette *et al.,* 2003). Therefore, extrapolating the data obtained to the *in vivo* situation is limited. As a result, a limited study to was undertaken using a commercially available stratified model mucosa (SkinEthic), that has previously been used for screening the compatibility of new pharmaceutical products and dental materials and to examine the response of the epithelium to infection (de Brugerolle de Fraissinette *et al.,* 1999; Vannet *et al.,* 2007).

8.2.1 Cell Viability:

The MTT assay was used as an initial approach to evaluate the response of RHGE and RHOE to CsA and bacterial products. CsA had no effect on tissue viability of both RHGE and RHOE, whilst the bacterial supernatants caused a tissue dependent- effect. RHGE viability was decreased after treatment with P.g, A.a and P.i whilst all bacterial supernatants increased RHOE viability. Furthermore, combinations of CsA plus any of the bacterial supernatants increased the viability of RHGE with the most pronounced effect being found with CsA/F.n where the level of viability was significantly higher than control. However, all combinations had no effect on RHOE viability. The scope of MTT assay as mentioned above is limited to the cell viability without providing an idea on the cellular pathways involved, Therefore more sophisticated studies were also required to evaluate the cellular response to CsA and bacterial products and whether this response may involve a modulation in the local inflammatory response.

8.2.2 Cell proliferation:

The multiparameter FACS analysis used to assess cell proliferation and turnover was not applicable to the stratified cultures and therefore a qPCR approach was used to investigate CCND1 and CCNB1 gene expression that encodes cyclin D1 and cyclin B1 respectively.

CsA alone had no effect on CCND1 expression in RHGE, whilst a significant increase was seen cultures exposed to all of the bacterial supernatants. This increase in CCND1 expression was further enhanced by addition of CsA. Conversely, a significant reduction in CCNB1 expression was observed in RHGE cultures exposed to CsA alone or all bacterial supernatants. Interestingly, exposure of RHGE cultures to combinations of CsA and any of bacterial products caused a reversal of the inhibitory effect with the most marked change being with A.a and P.i.

Thus, our results indicate that the exposure to bacterial products caused a bimodal effect on cyclin expression in RHGE; while they stimulated CCND1 expression which, in turn, accelerates the entry of cell cycle machinery into S phase (Tashiro *et al.,* 2007). They arrested the cell cycle at G2/M by down-regulating the CCNB1 expression and consequently inhibition of the cellular mitosis. This later effect may

explain at least partly the decrease in RHGE viability determined by MTT assay after the exposure to bacterial products.

RHOE cultures reacted differently to bacterial products where an up-regulated expression of CCNB1 and CCND1 was only observed in cultures exposed to F.n and A.a supernatants respectively. CsA had no significant effect on expression of these genes whilst the combination of CsA and all of the bacterial supernatants caused a significant reduction in CCNB1 expression but had no effect on CCND1.

The reasons behind the differences in the response to CsA and bacterial products between the RHGE and RHOE in the present study is not fully understood but it might partly be related to the differences in the keratinisation level of each tissue or the different turnover rate between these cultures (Carranza, 1990).

However, this bi-model and gingival region-dependent effect of bacterial products on the proliferative activity of epithelium (stimulatory on RHOR and inhibitory on RHGE) observed in our study may interpret the discrepancy in studies on the effect of gingival inflammation on the dividing activity of gingival keratinocytes; Celenligil *et al* (2000) showed that the proliferative activity of the inflamed oral gingival epithelium was significantly higher compared to that in healthy gingival. Conversely, Carro *et al* (1997) showed that the proliferation rate of keratinocyte cultures derived from clinically healthy to slightly inflamed human gingival tissue was much higher than that of cultures derived from severely inflamed gingiva.

The increase in the proliferative activity of RHGE exposed to combinations of CsA and bacterial products is consistent with the clinical observation of CsA-induced gingival hyperplasia being confined to the keratinized gingiva (Friskopp & Klintmalm, 1986) and further supported by an animal study revealing that this condition was more prominent in the buccal gingiva (Spolidorio *et al.,* 2001). These findings are also supported by earlier *in vivo* study in which CsA-induced gingival hyperplasia was associated with hyperproliferation mainly in the oral gingival epithelium. In this study Nurmenniemi *et al* (2001) attributed the lack of proliferative activity in the sulcular epithelium to direct contact with the dental plaque or inflammation (Carro *et al.,* 1997) masking the proliferative effect of CsA.

Our study was also partly consistent with an animal study that found an increase in the proliferative activity mainly in the buccal and to a lesser extent in the sulcular epithelium of rats subjected to CsA treatment along with ligature-induced gingival inflammation suggesting that CsA-induced gingival hyperplasia might be mediated by an increase in the proliferative activity of epithelium cells and that dental plaque might aggravate the condition (Cetinkaya *et al.,* 2006).

In light of our findings, it appears that CsA does not exert a direct effect on the proliferative activity of keratinocytes but instead, the hyper-proliferative activity observed in CsA-induced gingival hyperplasia may be an indirect consequence of drug interactions with other intrinsic or extrinsic factors.g., modulation of the local inflammatory response and cytokine release. Consequently, the different pattern of response between RHGE and RHOE may reflect the complexity in the direct and indirect effect of CsA on proliferation of keratinocytes.

8.2.3 Pro-inflammatory cytokines:

Low levels of IL-1α and IL-6 were released from RHOE and RHGE cultures and CsA and bacterial supernatants alone or in combination did not modulate their release. IL-8 release in RHOE cultures was much higher than that in RHGE cultures. A.a, Pi and F.n stimulated IL-8 release from RHGE whilst all 4 bacteria stimulated release from RHOE. CsA alone caused a significant reduction in IL-8 release from RHOE but not RHGE whilst CsA in combination with all bacterial supernatants inhibited the release of IL-8 from both RHGE and RHOE. The finding that IL-8 release was upregulated by the bacterial supernatants is in contrast to the results seen with the monolayer cultures. The reason for this is unclear, but may reflect subtle interactions within stratified epithelium that would not be present in monolayer cultures. The increase is however, perhaps not surprising as IL-8 is a potent neutrophil chemoattractant and significance of acute inflammation in gingivitis and periodontitis (Bickel, 1993; Tsai *et al.,* 1995). CsA inhibition of IL-8 release has been reported in psoriatic lesions (Prens *et al.,* 1995) and so it was perhaps surprising that CsA alone had no effect in RHGE. This may however, be related to the low levels released as inhibition was present after stimulation with bacterial supernatant. Acute inflammation leads to changes in the tissue (Tsai *et al.,* 1995) and whether the abrogation of the stimulatory

effect of bacterial products on IL-8 release exerted by CsA may play a role in hyperplasia requires further investigation.

The absence of an effect on IL-6 release was in contrast with the effect seen in the monolayer keratinocytes cultures. An increased expression of IL-6, IL-8 and IL-1β has also been reported in engineered human oral mucosa exposed to P.g (Andrian *et al.,* 2004). This study, however, used a suspension of live bacteria. In addition, the type of mucosa used in their study was different from ours as they used a full-thickness mucosa that included the epithelial/connective tissue interaction.

Furthermore, our study did not find a positive correlation between cell viability and release of any of the cytokines studied. These findings were further supported by an early *in vitro* study that found that the release of IL-1a and IL-8 in reconstructed human epidermis was not correlated with cell viability or cytotoxicity, but to the nature of agent applied. (Coquette *et al.,* 1999). While IL-1a release served as a good parameter for irritancy, release of IL-8 was linked to sensitizing potency (Coquette *et al.,* 2003). These findings reflect the complexity of molecular pathways underlying the cytokines expression and imply interactions with various cellular targets (Coquette *et al.,* 1999).

The differences in the findings between monolayer cultures and stratified cultures may be attributed to the different culture system as the multilayered nature of the mucosa along with numerous tight junctions may limit the agent diffusion through the tissue layers. An additional protection may also be provided by the superficial layers when the epithelium is keratinized. (Gaballah *et al.,* 2007; Gaballah *et al.,* 2008). Therefore, the lack of this structure renders the monolayer cultures more susceptible to the drugs where doses triggering responses are usually several folds of magnitude lower than those which provoke biological reactions *in vivo* (Coquette *et al.,* 2003). Thus, upon direct exposure to non-physiological concentrations of agents, cell damage seems to occur more rapidly in monolayer cultures compared to their counterpart in multilayered cultures (Tomakidi *et al.,* 2000). Therefore, results obtained from monolayer culture tests are normally difficult to be interpreted or extrapolated to the *in vivo* situation (Welss *et al.,* 2004).

8.2.5 Apoptosis:

There was no significant alteration in either Bax/Bcl-2 gene ratio or Fas-L gene in any of the treatment groups. However, due to the limitations of our study that included a limited number of samples for each treatment group in addition to the limited key apoptotic mediators involved in our study, more investigations are required in this regard to obtain conclusive findings whether the dysregulated apoptosis may be involved in pathogenesis of the gingival changes associated with CsA.

However, Buduneli *et al* (2007) minimized the importance of dental plaque in pathogenesis of CsA-induced gingival hyperplasia suggesting that that the dysregulation of tissue homeostasis observed in CsA-induced gingival hyperplasia could be attributed to the independent effect of CsA rather than to the influence of chronic gingival inflammation. In this study they found that that CsA-hyperplastic gingiva had significantly less numbers of apoptotic cells compared to the inflamed gingival. However, one limitation when compare our results with theirs is that our study was *in vitro* versus their *in vivo* study that involves a complex of microorganisms.

8.3 Conclusion and Future work

The results of the monolayer cultures suggest that CsA, at a clinically relevant dose, appears to modulate cytokine release and proliferative activity by gingival cells. There was also clear evidence that combinations of CsA and P.i or A.a caused marked changes in the proliferative activity of the cells. Although these findings suggest that they may have an important role in CsA-associated gingival changes, further studies are necessary to confirm the results. These results were supported by the stimulatory effect of CsA and bacterial supernatants on proliferation of the model RHGE. Interestingly the changes seen in cytokine release by monolayers were not evident in the stratified cultures. This may reflect the fact that the models used in this study exclusively consist of keratinocytes and lack Langerhans cells, which play a fundamental role in initiating the cutaneous immune response and cytokine expression (Srivastava *et al.,* 1994; Cumberbatch *et al.,* 1996), in addition to absence of the leukocytes diminishing the complexity of the observable cytokine milieu (Vannet *et al.,* 2007). Furthermore, the absence of mesenchyme-keratinocyte interaction may mask the complexity of the cellular interactions observable *in vivo*. Further studies involving co-cultures of fibroblast and the model mucosa are therefore needed to establish the significance of these changes.

Taken together these results, outlined in this thesis suggest that CsA may affect the gingival fibroblast and keratinocytes both directly and indirectly via modulation of the cytokine release and the host response to bacterial products indicating a potential role of bacterial products in pathogenesis of gingival hyperplasia.

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Chapter Nine
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Chapter Ten
Appendices

Appendix 1
Determination of bacterial supernatant toxicity

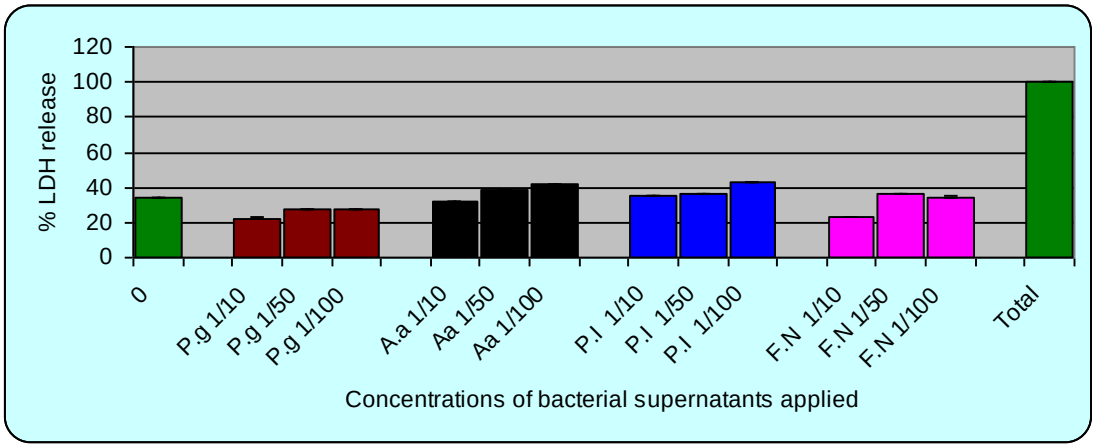

Figure A1. LDH release from OKF6. Cell lines were challenged for 24h with ascending doses of bacterial supernatants. Extracellular release of LDH was then measured using a Cytotoxicity Detection Kit^{plus}. Two controls were used in the study; high control which represents the totally lysed cells and low control which is medium containing un-treated cells. Results are expressed as percentage of total LDH. Bars represent Mean ± SEM of 12 samples.

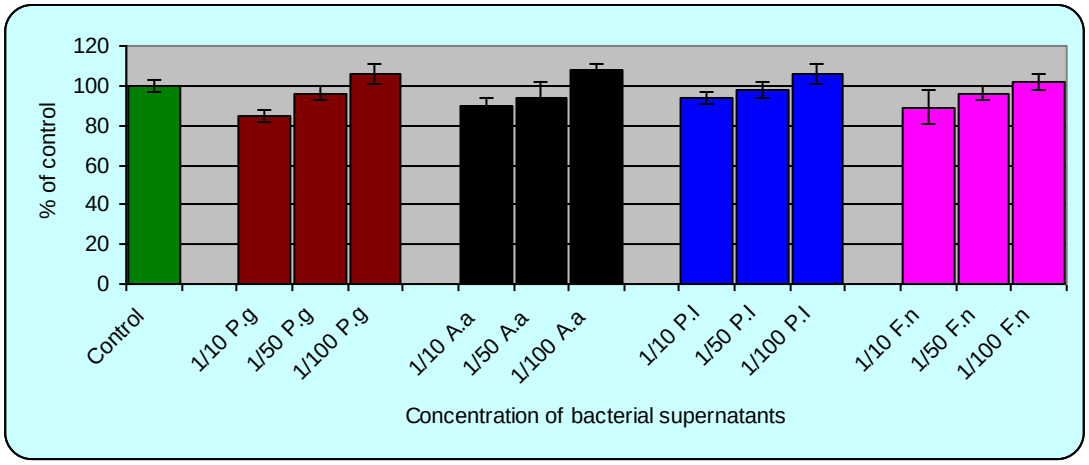

Figure A2. FIBS viability. Cultures were stimulated for 24h with ascending concentrations of bacterial supernatant. Cell viability was measured by Alamar blue. Untreated cells were served as control. Results are expressed as the Mean ± SEM (n=9). Statistical significance was regarded when $p < 0.05$ compared to control.

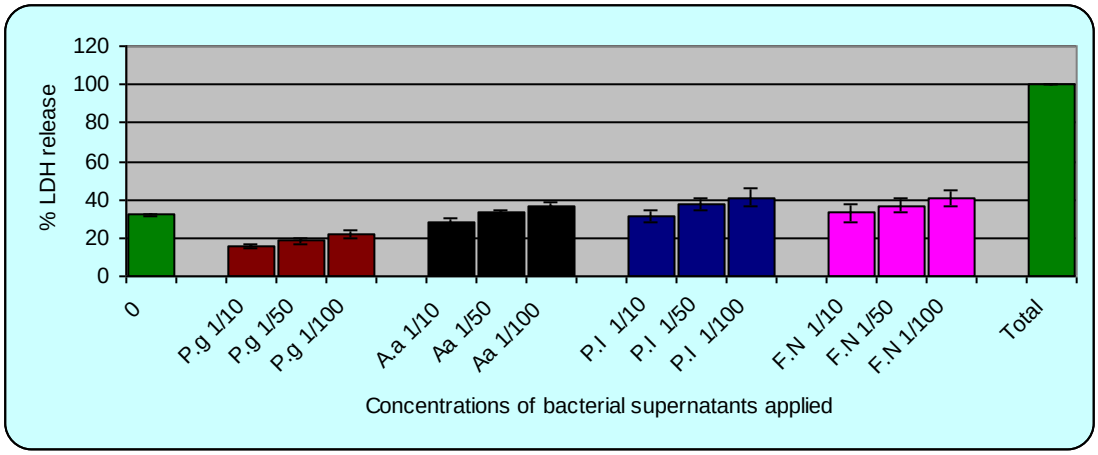

Figure A3. LDH release from TR146. Cell lines were challenged for 24h with ascending doses of bacterial supernatants. Extracellular release of LDH was then measured using a Cytotoxicity Detection Kit^{plus}. Two controls were used in the study; high control which represents the totally lysed cells and low control which is medium containing un-treated cells. Results are expressed as percentage of total LDH. Bars represent Mean ± SEM of 12 samples.

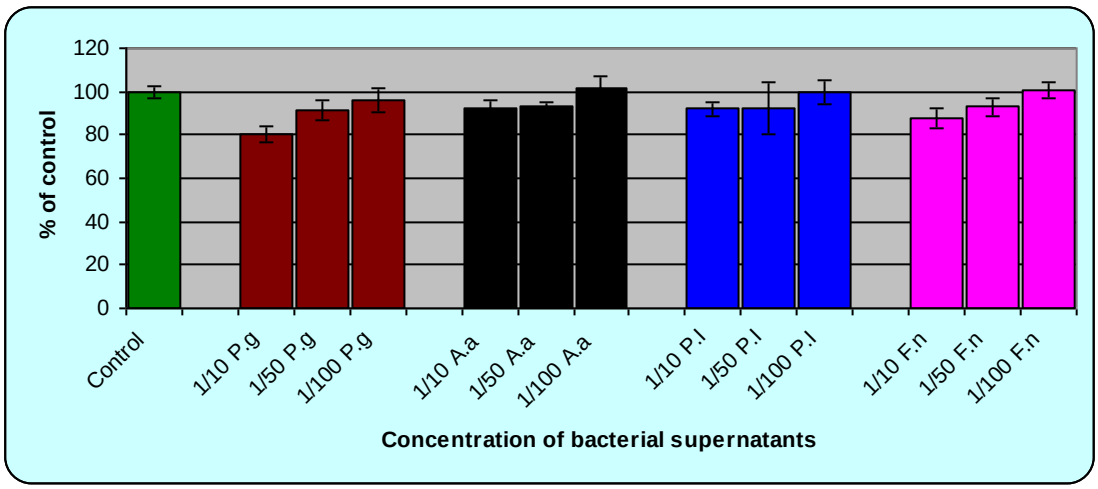

Figure A4. FIBS viability. Cultures were stimulated for 24h with ascending concentrations of bacterial supernatant. Cell viability was measured by Alamar blue. Untreated cells were served as control. Results are expressed as the Mean ± SEM (n=9). Statistical significance was regarded when $p < 0.05$ compared to control.

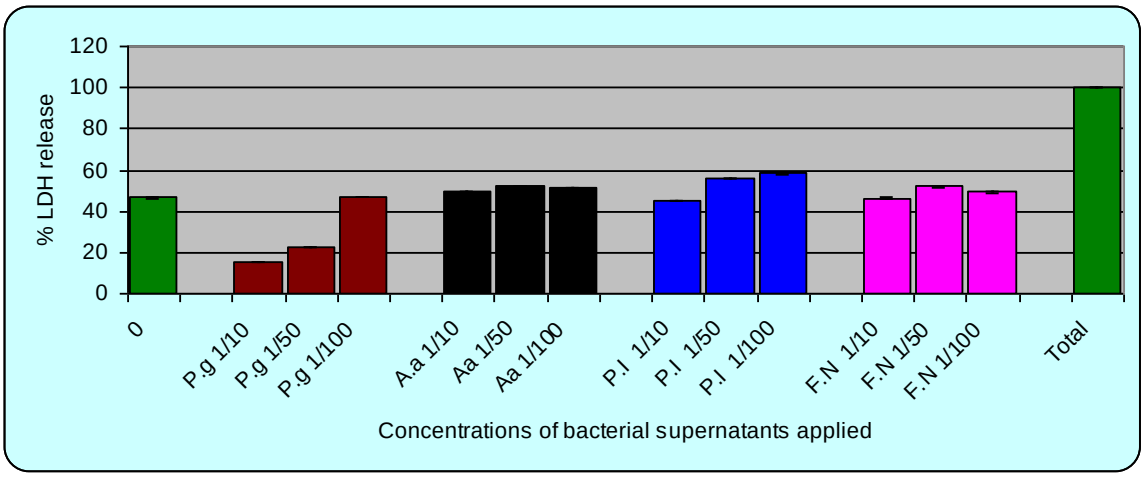

Figure A5. LDH release from HGF. Cell lines were challenged for 24h with ascending doses of bacterial supernatants. Extracellular release of LDH was then measured using a Cytotoxicity Detection Kit^{plus}. Two controls were used in the study; high control which represents the totally lysed cells and low control which is medium containing un-treated cells. Results are expressed as percentage of total LDH. Bars represent Mean ± SEM of 12 samples.

Appendix 2
FACS plots

List of abbreviations:

CsA……..Cyclosporin-A (250 & 2000ng/ml)
P.g ……...P. gingivalis
A.a……..A. actinomycetemcomitans
P.i…… P. intermedia
F.n……F. nucleatum

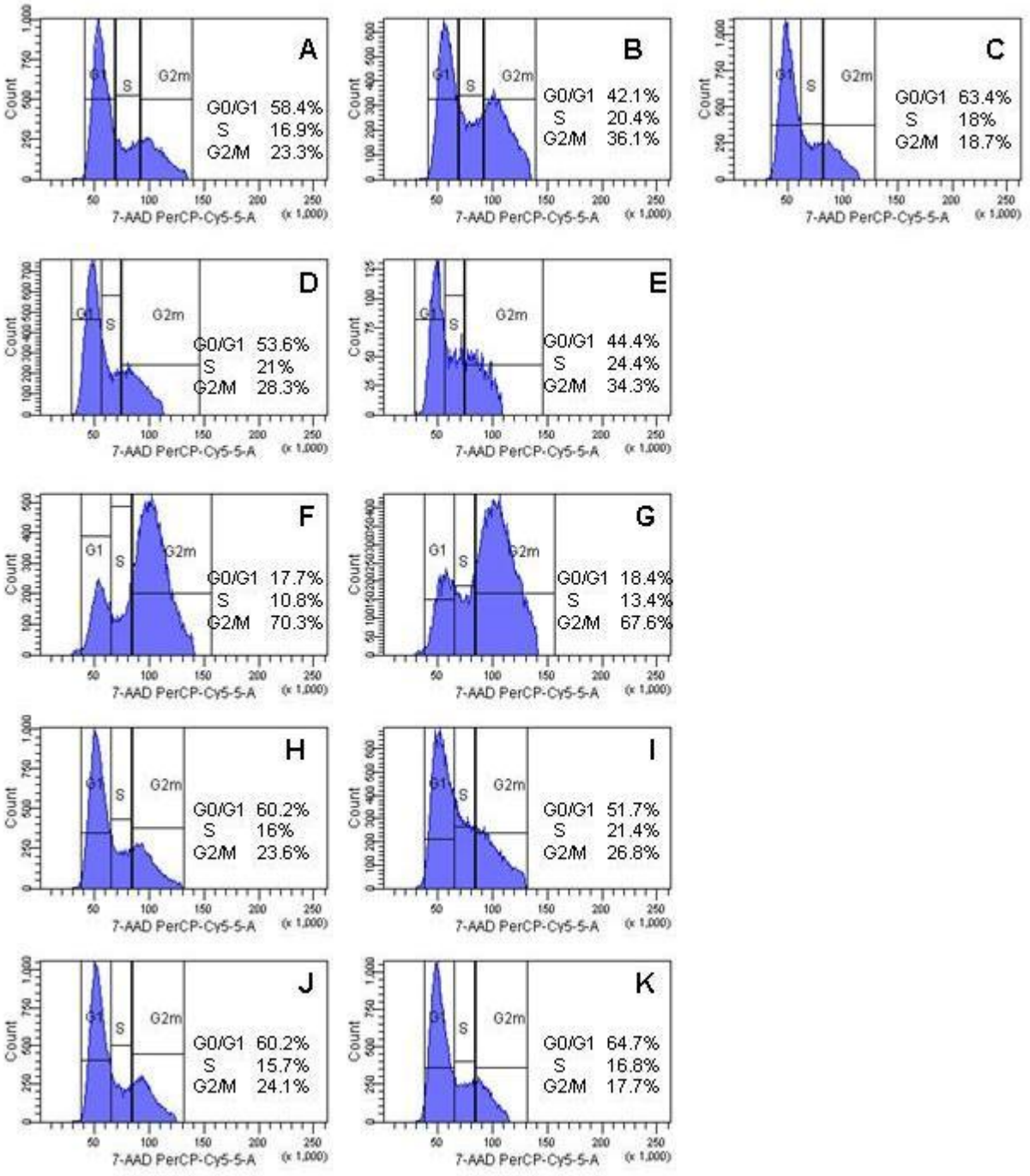

Fig A1. Single parameter histograms of DNA content displaying the cell cycle distribution of FIBS after 24h of exposure to a combination of CsA and bacterial supernatant. A; serum starved **B;** serum rechallenged **C;** 2000ng/ml CsA. **D;** P.g. **E;** P.g plus 2000ng/ml CsA **F;** A.a. **G;** A.a plus 2000ng/ml CsA. **H;** P.i. **I:** P.i plus 2000ng/ml CsA, **J:** F.n. **K:** F.n plus 2000ng/ml CsA.

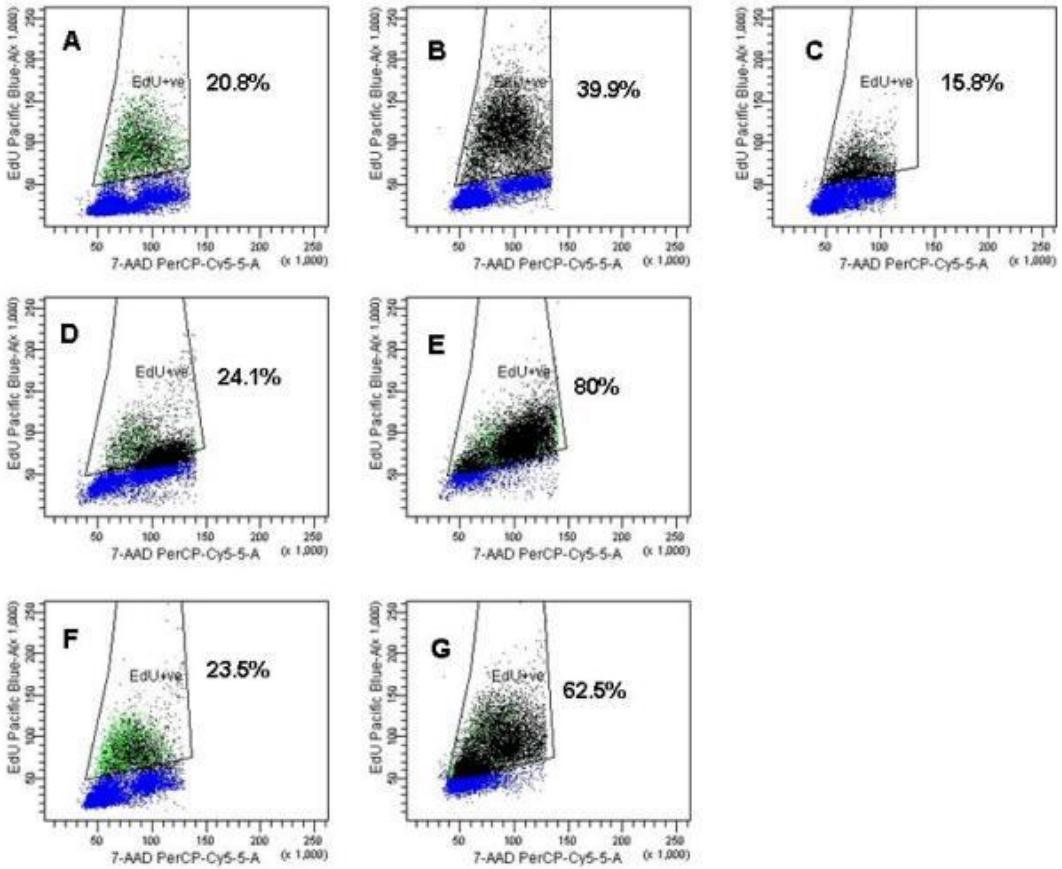

Fig A2. Dual parameter dot plots analysing the Edu positive cells against DNA content in FIBS after 24h of exposure to a combination of CsA and bacterial supernatants. Results were expressed as a % of positive cells incorporating Edu.
A. Serum-starved. **B**; Serum-rechallenged. **C**; 2000ng/ml CsA. **D**; A.a. **E**; A.a plus 2000ng/ml CsA. **F**; F.n. **G**; F.n plus 2000ng/ml CsA.

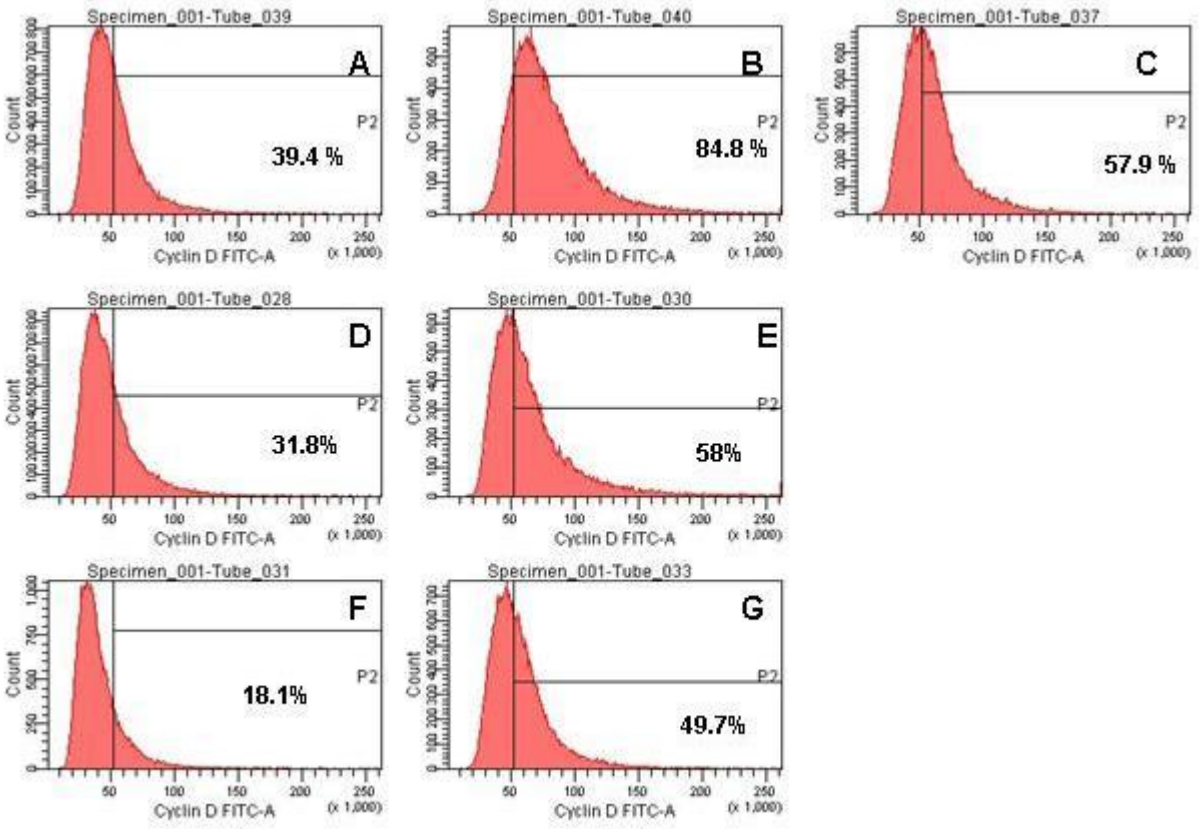

Fig A3. Flow cytometric analysis of cyclin D1 expression in FIBS cells after 24h exposure to a combination of CsA and bacterial supernatants.

Expression of cyclin D1 was analysed using single parameter histograms in: **A**. Serum-starved. **B**; Serum-rechallenged. **C**; 2000ng/ml CsA. **D**; P.i. **E**; P.i plus 2000ng/ml CsA. **F;** F.n. **G;** F.n plus 2000ng/ml CsA. Results were expressed as a % of positive cells expressing cyclin D1.

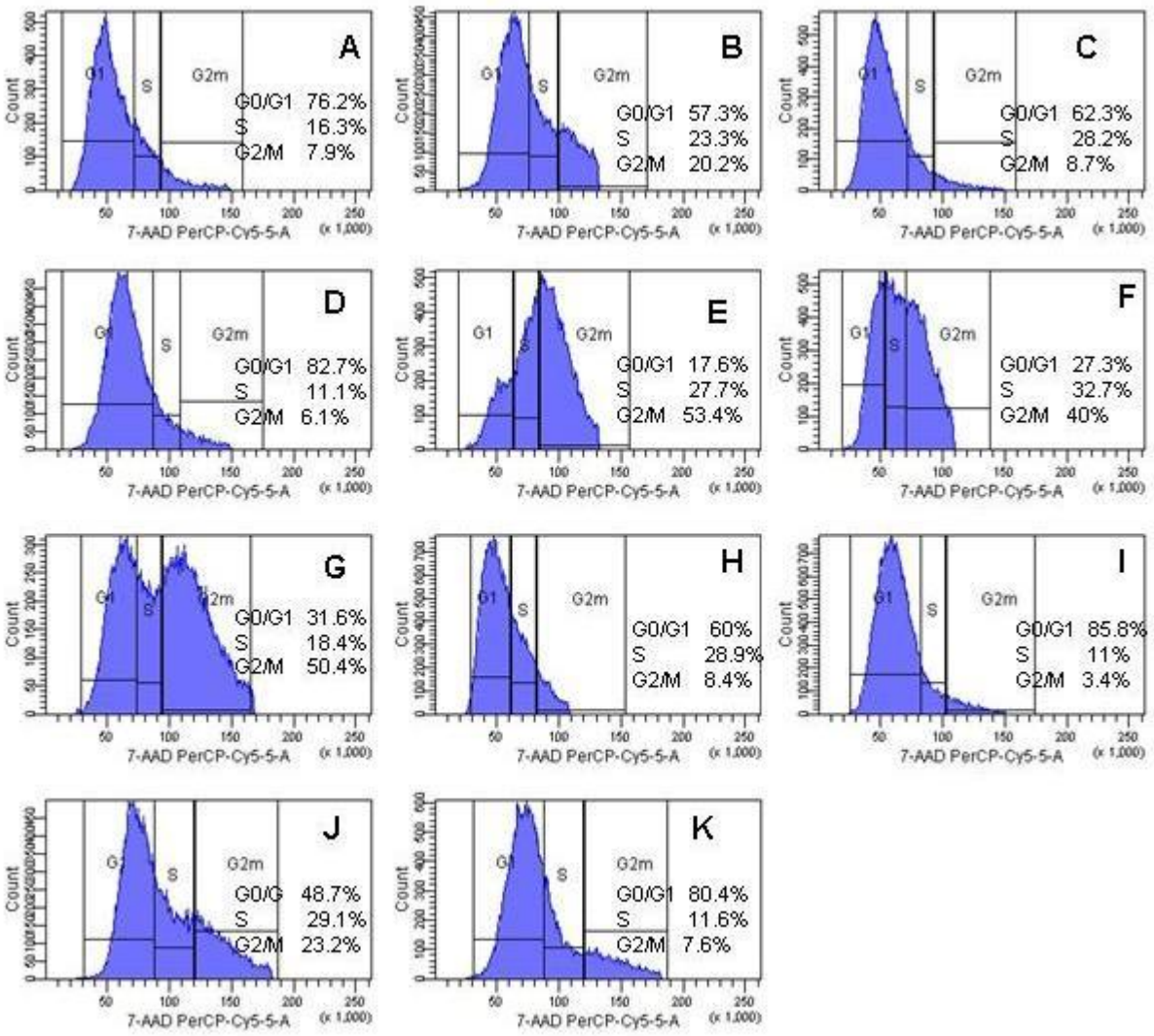

Fig A4. Single parameter histograms of DNA content displaying the cell cycle distribution of TR146 after 24h exposure to a combination of CsA and bacterial supernatants. A; serum starved. **B;** serum rechallenged. **C;** 250ng/ml CsA. **D;** 2000ng/ml CsA. **E**; A.a. **F;** A.a plus 250ng/ml. **G;** A.a plus 2000ng/ml. **H;** P.i. **I;** P.i plus 2000ng/ml CsA. **J:** F.n. **K:** F.n plus 2000ng/ml CsA.

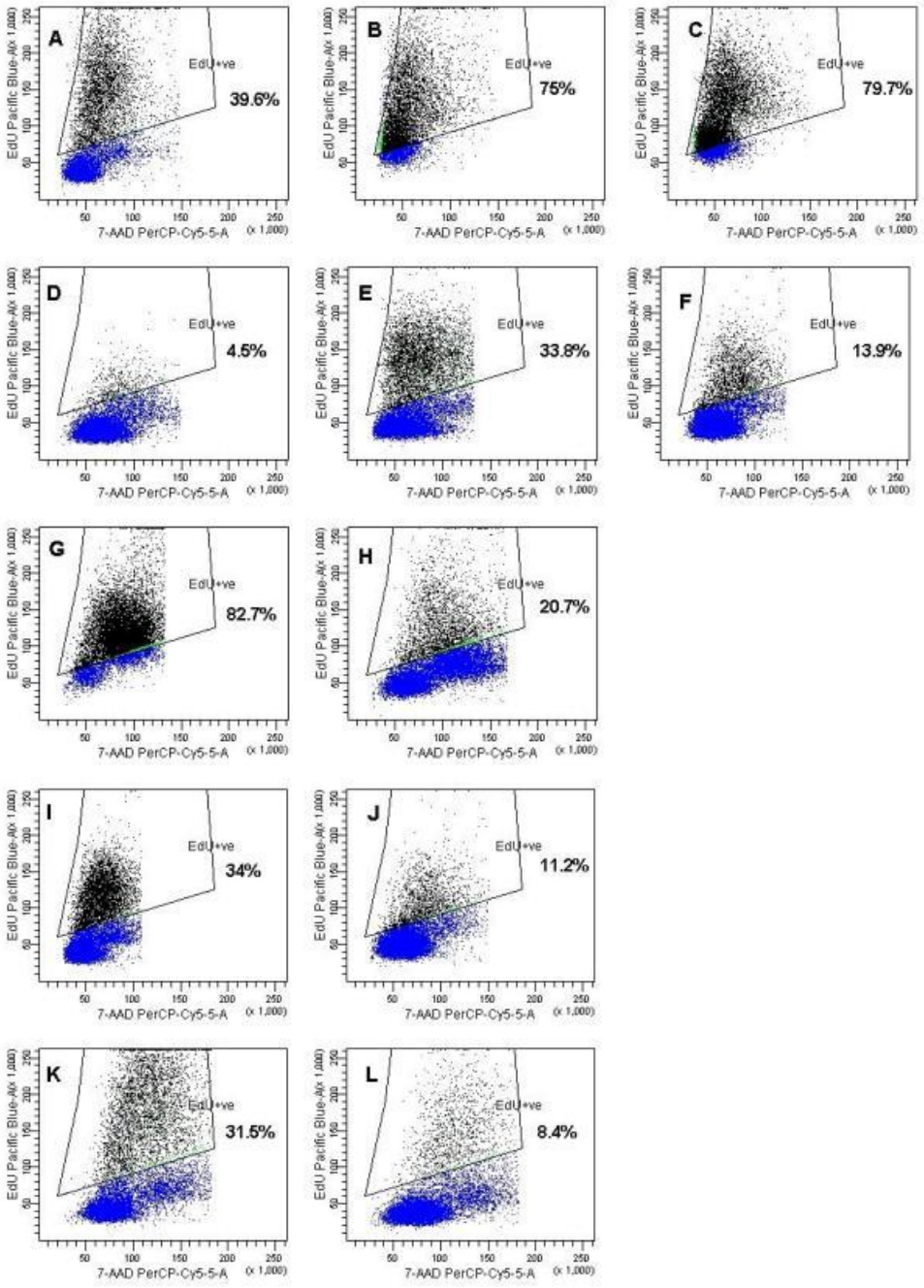

Fig A5. Dual parameter dot plots analysing the Edu positive cells against DNA content in TR146 after 24h of exposure to a combination of CsA and bacterial supernatants. Results were expressed as a % of positive cells incorporating Edu.

A; serum starved. **B;** serum rechallenged. **C;** 250ng/ml CsA. **D;** 2000ng/ml CsA. **E**; P.g. **F;** P.g plus 2000ng/ml. **G;** A.a. **H;** A.a plus 2000ng/ml CsA. **I;** P.i. **J:** P.i plus 2000ng/ml CsA. **K:** F.n. **L;** F.n plus 2000ng/ml CsA.

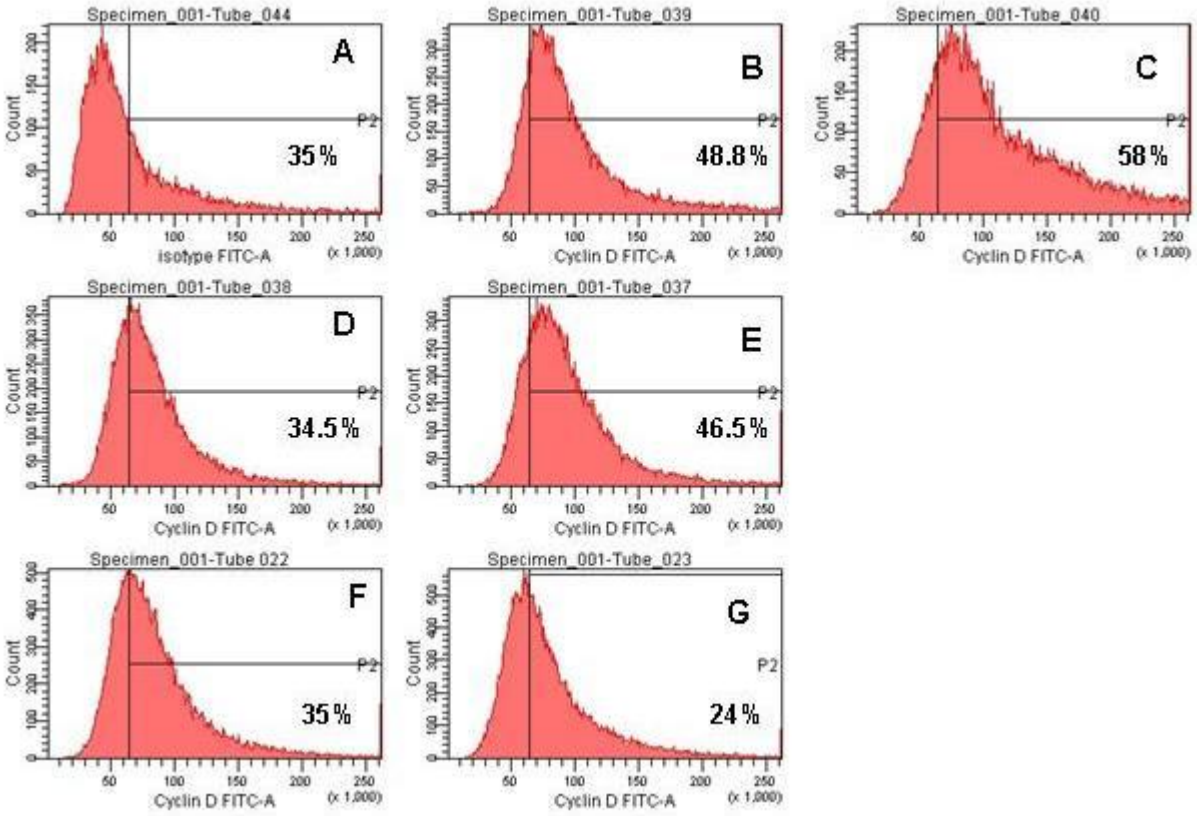

Fig A6. Flow cytometric analysis of cyclin D1 expression in TR146 cells after 24h exposure to a combination of CsA and bacterial supernatants.
Expression of cyclin D1 was analysed using single parameter histograms in: **A**. Negative isotype control showing a strong background by 35% that was afterwards deducted from values of all studied samples. **B;** Serum-starved. **C**; Serum-rechallenged. **D**; 250ng/ml CsA. **E**; 2000ng/ml CsA. **F;** P.g. **G**; P.g plus 250ng/ml CsA. Results were expressed as a % of positive cells expressing cyclin D1.

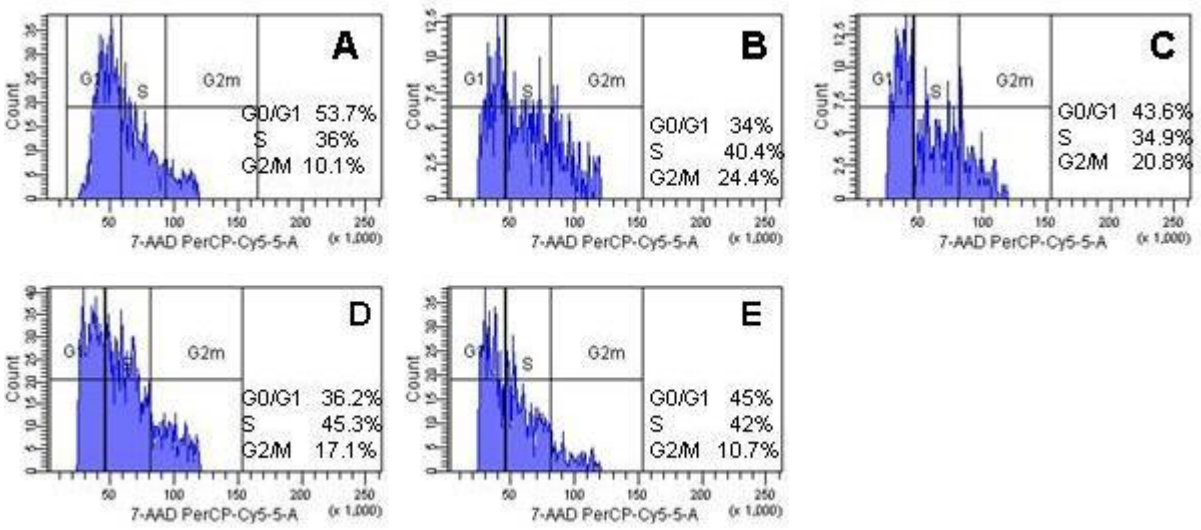

Fig A7. Single parameter histograms of DNA content displaying the cell cycle distribution of HGF after 24h exposure to a combination of CsA and bacterial supernatants. A; serum starved. **B;** serum rechallenged. **C;** 2000ng/ml CsA. **D;** F.n. **E;** F.n plus 2000ng/ml.

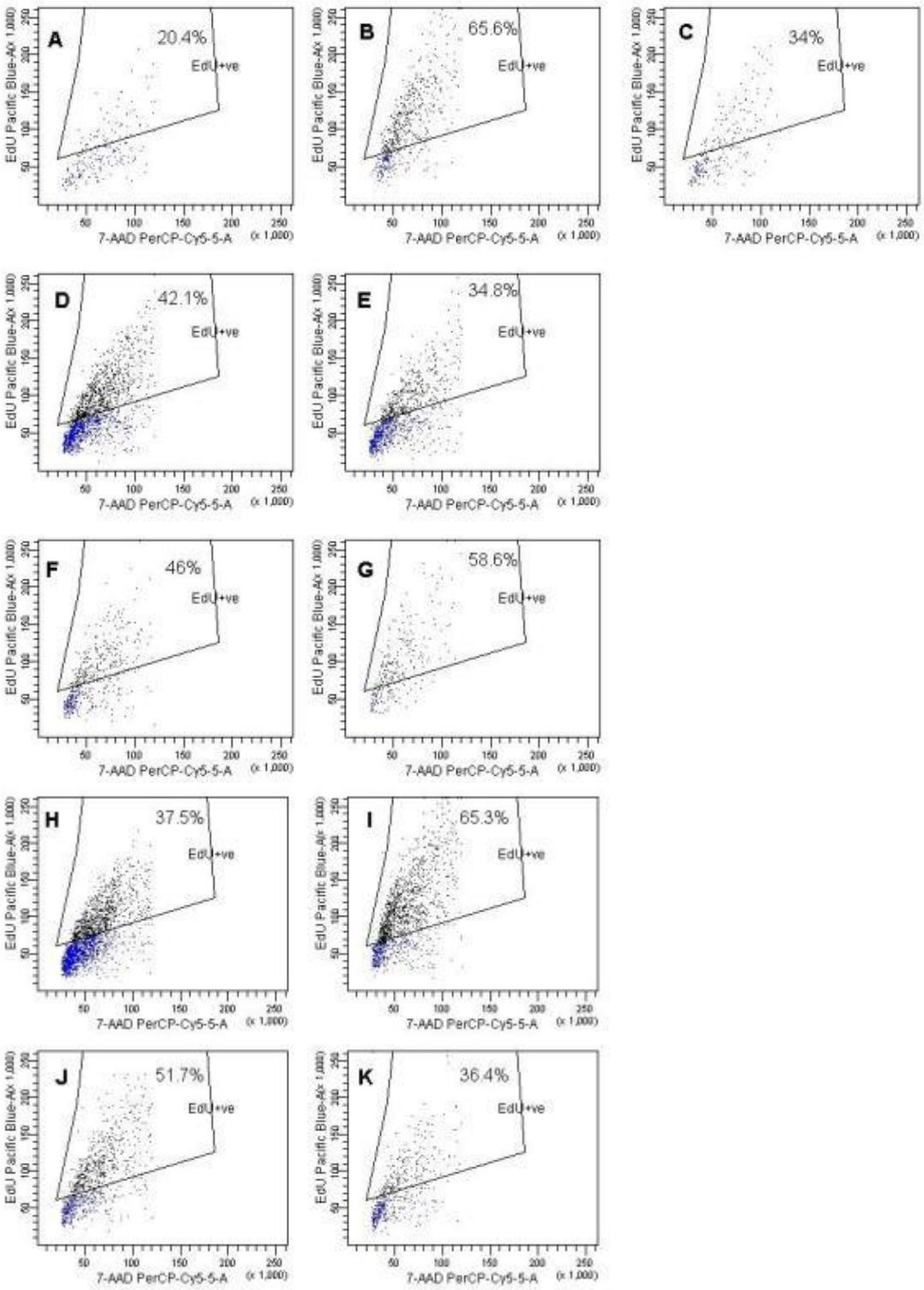

Fig A8. Dual parameter dot plots analysing the Edu positive cells against DNA content in HGF after 24h exposure to a combination of CsA and bacterial supernatants. Results were expressed as a % of positive cells incorporating Edu. **A**. Serum-starved. **B**; Serum-rechallenged. **C**; 2000ng/ml CsA. **D**; P.g. **E**; P.g plus 2000ng/ml CsA. **F**; A.a. **G**; A.a plus 2000ng/ml CsA. **H**; P.i. **I;** P.i plus 2000ng/ml CsA J; F.n. K; F.n plus 2000ng/ml CsA.

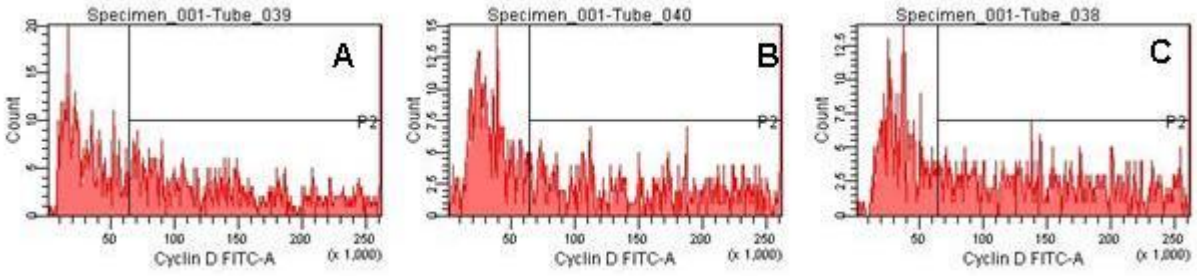

Fig A9. Flow cytometric analysis of cyclin D1 expression in HGF after 24h exposure to a combination of CsA and bacterial supernatants.

Expression of cyclin D1 was analysed using single parameter histograms in: **A**; Serum-starved. **B**; Serum-rechallenged. **C**; 2000ng/ml CsA. Results were expressed as a % of positive cells expressing cyclin D1. Results were expressed as a % of positive cells expressing cyclin D1.

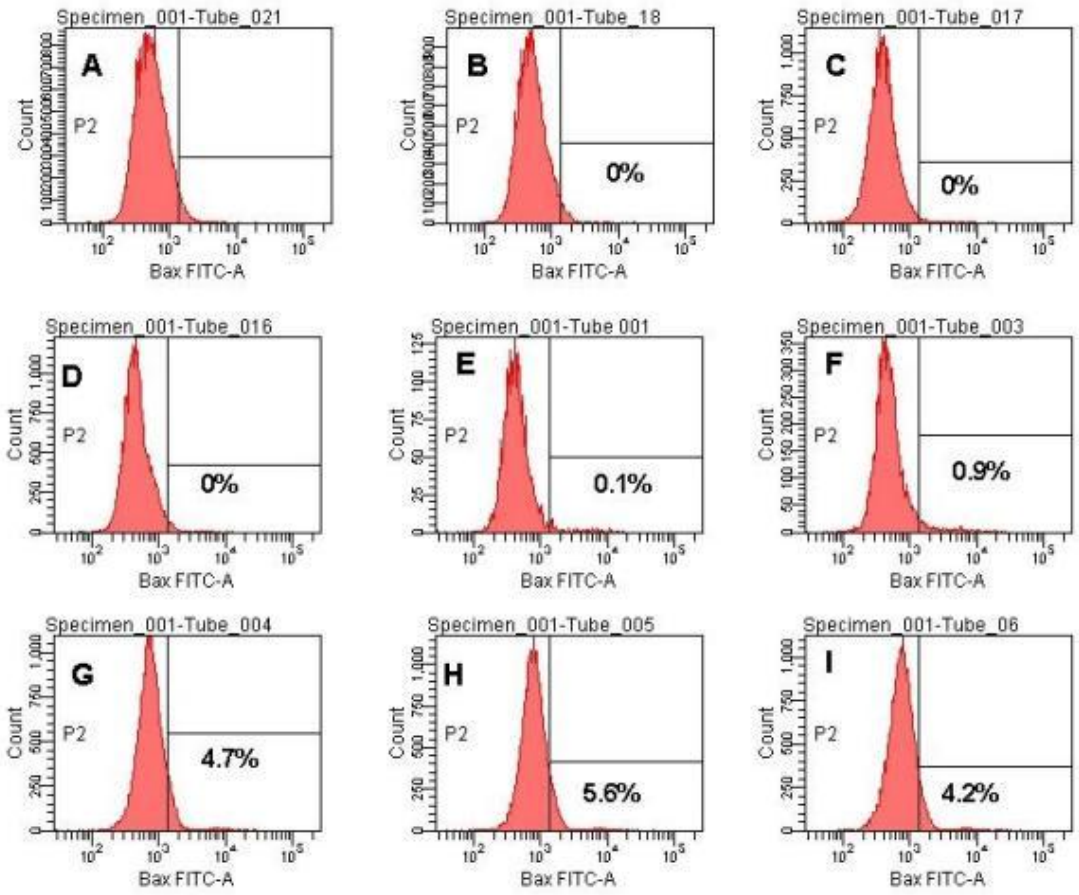

Fig A10. Flow cytometric analysis of Bax expression in FIBS cells after 24h exposure to a combination of CsA and bacterial supernatants.

Expression of Bax was analysed using single parameter histograms in: **A**; Negative isotype control. **B**; untreated FIBS (control). **C**; 250ng/ml CsA. **D**; 2000ng/ml CsA. **E;** P.g. **F;** P.g plus 2000ng/ml CsA. **G;** A.a. **H;** A.a plus 250ng/ml CsA. **I;** A.a plus 2000ng/ml CsA. Results were expressed as a % of positive cells expressing Bax.

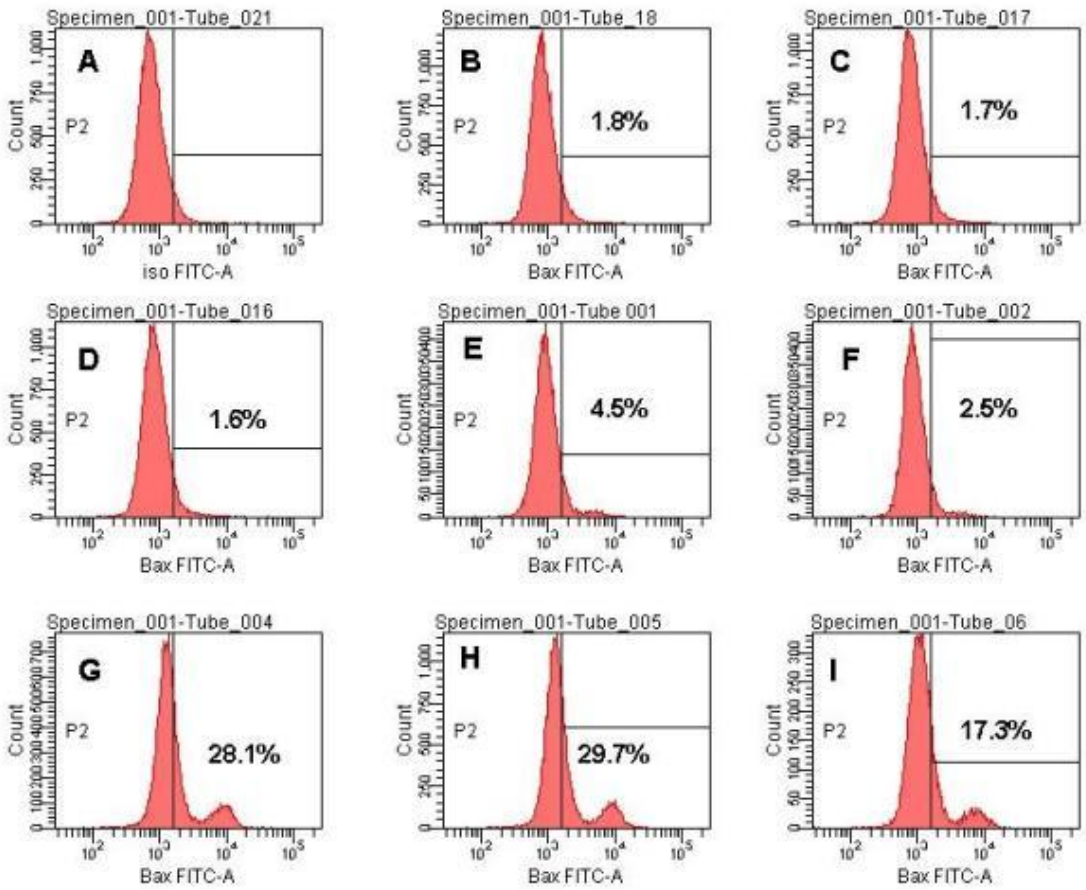

Fig A11. Flow cytometric analysis of Bax expression in OKF6 cells after 24h exposure to a combination of CsA and bacterial supernatants.

Expression of Bax was analysed using single parameter histograms in: **A**; Negative isotype control. **B**; untreated OKF6 (control). **C**; 250ng/ml CsA. **D**; 2000ng/ml CsA. **E;** P.g. **F;** P.g plus 250ng/ml CsA. **G;** A.a. **H;** A.a plus 250ng/ml CsA. **I;** A.a plus 2000ng/ml CsA. Results were expressed as a % of positive cells expressing Bax.

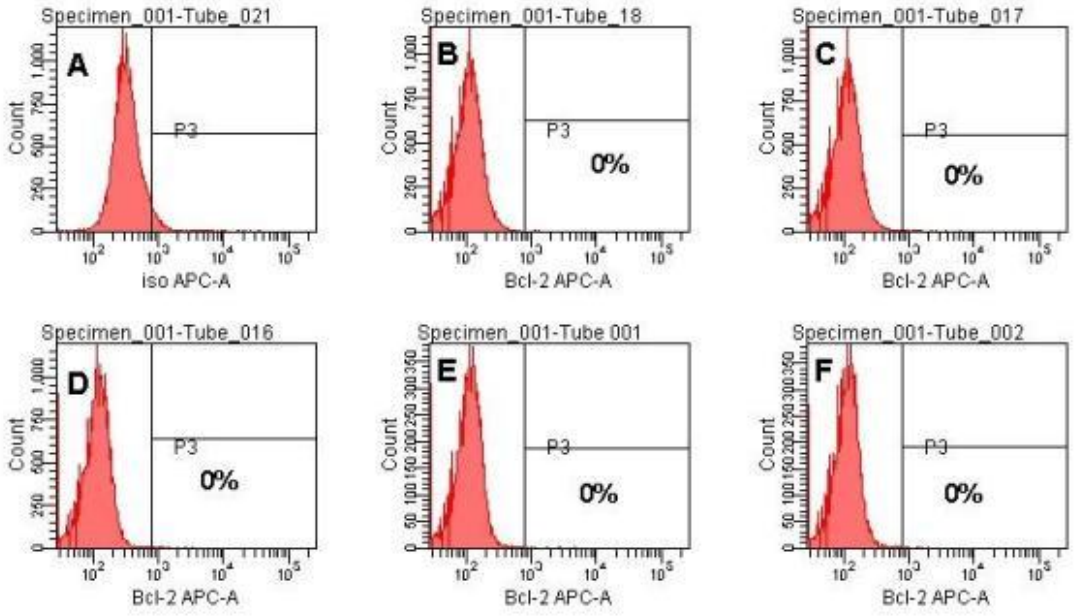

Fig A12. Flow cytometric analysis of Bcl-2 expression in OKF6 cells after 24h exposure to a combination of CsA and bacterial supernatants.
Expression of Bcl-2 was analysed using single parameter histograms in: **A**; Negative isotype control. **B**; untreated OKF6 (control). **C**; 250ng/ml CsA. **D**; 2000ng/ml CsA. **E;** P.g. **F;** P.g plus 250ng/ml CsA. Results were expressed as a % of positive cells expressing Bcl-2.

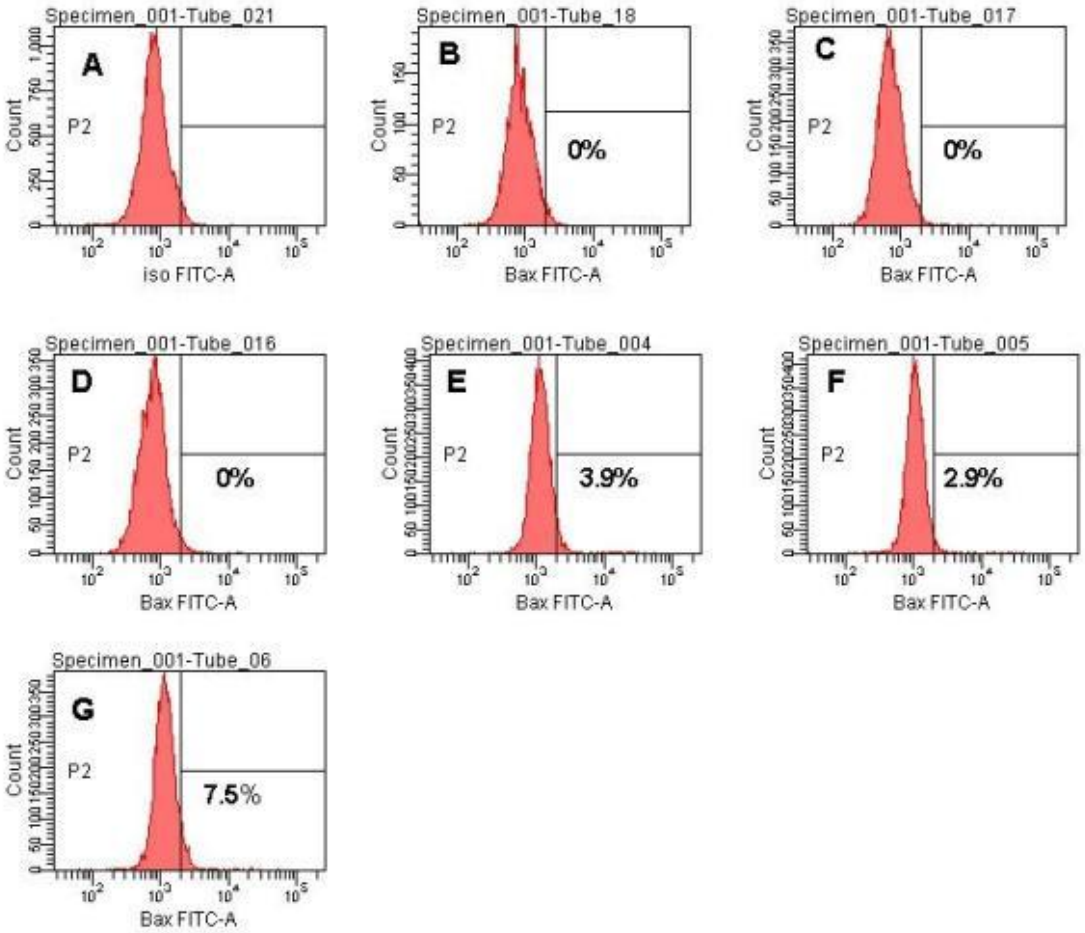

Fig A13. Flow cytometric analysis of Bax expression in TR146 cells after 24h exposure to a combination of CsA and bacterial supernatants.

Expression of Bax was analysed using single parameter histograms in: **A**; Negative isotype control. **B**; untreated TR146 (control). **C**; 250ng/ml CsA. **D**; 2000ng/ml CsA. **E;** A.a. **F;** A.a plus 250ng/ml CsA. **G;** A.a plus 2000ng/ml CsA. Results were expressed as a % of positive cells expressing Bax.

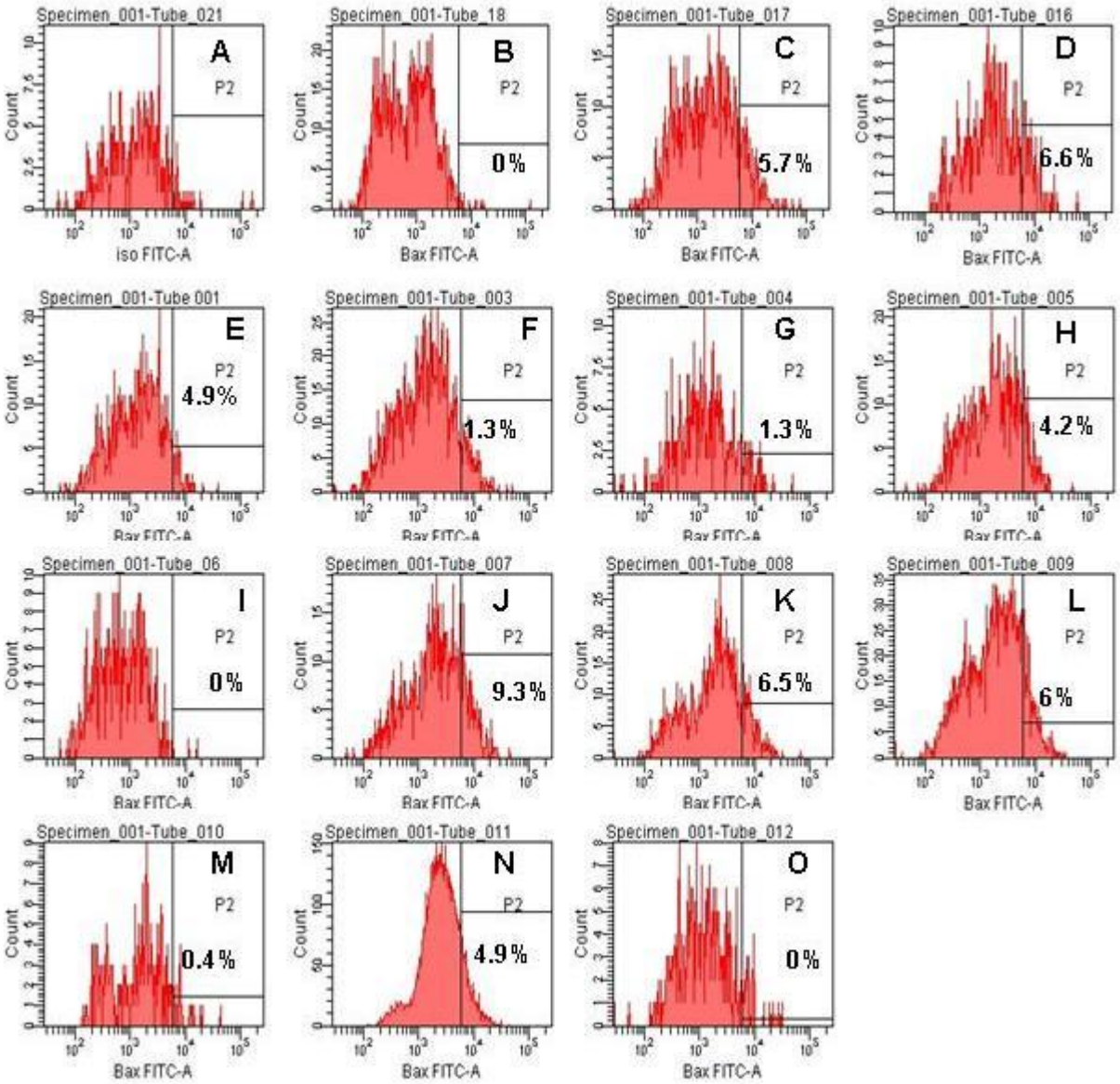

Fig A14. Flow cytometric analysis of Bax expression in HGF cells after 24h exposure to a combination of CsA and bacterial supernatants.

Expression of Bax was analysed using single parameter histograms in: **A**; Negative isotype control. **B**; untreated HGF (control). **C**; 250ng/ml CsA. **D**; 2000ng/ml CsA. **E;** P.g. **F;** P.g plus 2000ng/ml CsA. **G;** A.a. **H;** A.a plus 250ng/ml CsA. **I;** A.a plus 2000ng/ml CsA **J;** P.i. **K;** P.i plus 250ng/ml CsA. **L;** P.i plus 2000ng/ml CsA. **M;** F.n. **N;** F.n plus 250ng/ml CsA. **O;** F.n plus 2000ng/ml CsA. Results were expressed as a % of positive cells expressing Bax.