EVALUATION OF EXPRESSION OF B7-H3 IN GINGIVAL FIBROBLASTS - A CELL CULTURE, IMMUNOCYTOCHEMISTRY AND FLOWCYTOMETRY STUDY

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EVALUATION OF EXPRESSION OF B7-H3 IN GINGIVAL FIBROBLASTS - A CELL CULTURE, IMMUNOCYTOCHEMISTRY AND FLOWCYTOMETRY STUDY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. KV. ARUN, MDS.,** Professor, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

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CERTIFICATE

This is to certify that this dissertation titled "**EVALUATION OF EXPRESSION OF B7-H3 IN GINGIVAL FIBROBLASTS – A CELL CULTURE, IMMUNOCYTOCHEMISTRY AND FLOWCYTOMETRY STUDY**" is a bonafide record of work done by **Dr. Nikhita Rebekah David** under my guidance during the study period of **2013-2016**.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATIONS

1. APC	-	Antigen Presenting Cell
2. APES	-	3 amino- propyl tri-ethoxysilane
3. BCR	-	B Cell Receptor
4. BOP	-	Bleeding on Probing
5. CAL	-	Clinical Attachment Loss
6. CD	-	Cluster of Differentiation
7. CD40L	-	CD40 Ligand
8. CRS	-	Chronic Rhinosinusitis
9. CTLA-4	-	Cytotoxic T-Lymphocyte Antigen -4
10. DAB	-	Diamino benzidine
11. DC	-	Dendritic cell
12. DIGO	-	Drug Induced Gingival Overgrowth
13. DPX	-	Di-n- butyl phthalate in Xylene
14. EDTA	-	Ethylene diamino tetraacetic acid
15. GF	-	Gingival fibroblasts
16. HSS-HRP	-	High Sensitivity Streptavidin conjugated to Horse Radish Peroxidase
17. ICOS	-	Inducible costimulatory
18. IFN-γ	-	Interferon – gamma
19. IL	-	Interleukin

20. LI	-	Labeling Index
21. LPS	-	Lipopolysaccharide
22. MHC	-	Major Histocompatibility Complex
23. NF-κB	-	Nuclear Factor Kappa B
24. PBS	-	Phosphate buffered saline
25. PD	-	Probing Depth
26. PD-L	-	Programmed Cell Death-1 Ligand -1
27. PD-L2	-	Programmed Cell Death -1 Ligand -2
28. PDLF	-	Periodontal Ligament Fibroblasts
29. RCC	-	Renal Cell Carcinoma
30. TCR	-	T Cell Receptor
31. Th-1	-	Type 1 helper T cell
32. Th-2	-	Type 2 helper T cell
33. TREML-2 / TLT-2	-	Triggering Receptor Expressed on Myeloid Cell (TREM) Like Transcript-2

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Introduction

INTRODUCTION

Periodontal diseases are chronic inflammatory disorders characterized by the loss of the tooth supporting attachment apparatus. Although this chronic inflammation is evoked in response to the microbial antigens present in the subgingival biofilm, its severity is modified by several host related factors such as environmental factors and genetic or epigenetic traits.[101]

The inflammation begins as gingivitis before it progresses to involve the deeper structures of the periodontium. This progression is a process that is governed by a number of individual susceptibility factors that are yet to be fully understood. It is however reasonably clear that most of the tissue destruction occurring in periodontitis is a result of an exaggerated and non protective host response. [26, 167]

The immune responses in the periodontium are regulated by both the innate immune and adaptive immune cells which act in a coordinated and concerted manner. As inflammation becomes chronic, adaptive immune responses become important so as to afford protection against specific pathogens. The central role played by T cells in the etiopathogenesis of periodontal disease was recognized following the seminal work of Ivanher and Leanyi.[76]

T helper cells (Th) were delineated by Mossman and Coffman[118] into (Th1) and Th2 based on their pattern of cytokine secretion. It is now well

recognized that T helper cells play a central role in modulating both arms (humoral and cell mediated) of the immune responses. [137]

T cell activation depends on the presence of APCs (Antigen Presenting Cells) and requires two signals- a primary signal generated by the antigen and a secondary co stimulatory signal. In the absence of costimulation, T cells that encounter antigens either fail to respond and die by apoptosis or enter a state of unresponsiveness called anergy. Co stimulation ensures prevention of T cell apoptosis, release of the entire cytokine repertoire and thereby the effector functions of the T cells are maintained.[51,66,85,32,57] The best characterized co stimulatory pathway involves the interaction between CD28 present on the T cells and the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on the activated APCs.

The B7 family of molecules have been expanded in recent years to include B7-1, B7-2, ICOS ligand, B7-H1 (PD-L1), B7-DC (PD-L2), B7-H3 and B7-H4. All these proteins are transmembrane proteins consisting of two extracellular Ig-like domains, including an N-terminal, V-like domain and a membrane proximal C-like domain.

B7-H3 belongs to a new class of immune regulatory molecules, which function primarily in peripheral tissues, thereby modulating immune responses in target organs. Unlike other members of the B7 family, B7-H3 may be expressed in two different forms, with either one pair or two tandem pairs of V and C domains.

As the professional APCs like the dendritic cells, B cells and macrophages are capable of both antigen presentation as well as co stimulation, the entire repertoire of the B7 family is expressed in these cells. B7-H1 and B7-H2 are primarily expressed in the immune cells but B7-H3 is found to be expressed also on osteoblasts, fibroblasts, fibroblast-like synoviocytes, and epithelial cells. This broad expression pattern suggests diverse immunological and non immunological functions of B7-H3.[110]

In recent years, it has been suggested that fibroblasts may play a role in both the transition from acute to chronic inflammation and the persistence of chronicity**.** [24]

Gingival fibroblasts are responsible for the formation, maintenance and turnover of the extracellular matrix of the gingival connective tissue. In recent years, they have been proposed to play a secondary role in modulating the immune processes as well.[116]

Although the expression of CD40 in gingival fibroblasts and its interaction with T cell through the CD40-CD40L binding is well documented, it is not yet known if these cells express the newly discovered members of the B7 family of molecules.

Aims and Objectives

AIMS & OBJECTIVES

The aim of the present study was

- To characterize the gingival fibroblasts in cell culture and evaluate the expression of B7-H3 by immunocytochemistry
- To confirm and characterize the expression of B7-H3 in gingival fibroblasts by flowcytometry

Review of Literature

REVIEW OF LITERATURE

A historical evidence of periodontal disease:

Oral hygiene was practiced by the Sumerians of 3000 BC. The Babylonians and Assyrians apparently suffered from periodontal diseases and were believed to use various herbal medications.[64,79] Periodontal disease was the most common of all diseases found in the embalmed bodies of the Egyptians.[20, 141] Various herbal medicaments like honey, vegetable gum or residue of beer were prescribed for the strengthening of teeth and their supporting tissue.[48] Modern dentistry essentially only began in the latter half of the eighteenth century, particularly in France and England. Periodontics was considered as an individual branch only after association with deposits described as "tartar" on the teeth that lead to their exfoliation. Ever since then till the present, various aspects of periodontal disease have passed through different circles of perceptions with respect to its etiology and pathogenesis, not to mention a huge scope in developing newer strategies for the treatment of the same.

Etiopathogenesis of periodontitis:

Periodontal diseases comprise a variety of conditions affecting the health of the periodontium.[162] by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both. It is

widely accepted that the initiation and progression of periodontitis are dependent upon the presence of microorganisms capable of causing disease.[115] Dental plaque is a complex microbial community containing an estimated 700 to 19,000 species/phylotypes.[55] However, studies have shown that dental plaque is essentially required but is not sufficient to induce periodontitis. It is the host immune response to this microbial challenge that ultimately can cause the destruction of the periodontium.[45] Therefore, the focus of interest shifted to understanding various aspects of the host's immune system to target various molecular mechanisms in the goal of battling periodontitis.

The immune system:

The physiologic function of the immune system is defense against infectious microbes. However, even noninfectious foreign substances can elicit immune responses. The two arms of the immune system are the innate arm and the adaptive arm. Innate immunity (also called natural or native immunity) provides the early line of defense against microbes. The principal components of innate immunity are physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces, phagocytic cells (neutrophils, macro phages) and natural killer (NK) cells, blood proteins, including members of the complement system and other mediators of inflammation and proteins called cytokines that regulate and coordinate many of the activities of the cells of innate immunity.[78] The primary response to pathogens in the innate immune system is triggered by

Pattern Recognition Receptors (PRRs) that bind Pathogen-Associated Molecular Patterns (PAMPs), found in a broad type of organisms. These receptor types include toll-like receptors, nucleotide-binding oligomerization domain (NOD) proteins, cluster of differentiation 14 (CD14), complement receptor-3, lectins and scavenger receptors.[3, 6]

In contrast to innate immunity, there are other immune responses that are stimulated by exposure to infectious agents and increase in magnitude and defensive capabilities with each successive exposure to a particular microbe. Because this form of immunity develops as a response to infection and adapts to the infection, it is called adaptive immunity. The cells that are involved in adaptive immune responses are antigen-specific lymphocytes, APCs that display antigens and activate lymphocytes, and effector cells that function to eliminate antigens. [56] There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity that are mediated by different components of the immune system and function to eliminate different types of microbes. Humoral immunity is the principal defense mechanism against extracellular microbes and their toxins and is mediated by B cells.[93] Cell mediated immunity is the principal defense mechanism against intracellular microbes such as viruses and some bacteria and is mediated by T cells.[142] The central role played by T cells in regulating immune response to periodontal disease have been well-documented.[49, 131, 144]

The Role of T cells:

Ivanher and Leanyi (1970) were the first to study the central role played by T cells in the etiopathogenesis of periodontal disease.[77] T cells were shown to be involved in various biologic functions.

Gemell et al (2007) described the role of T cells in the maintenance of homeostasis of periodontal tissues.[58]

Azuma M (2006) described the role of T cells in the modulation of inflammatory/immune responses.[10]

Teng et al (2000) described the role of T cells in mediating the bone loss that occurred during periodontal disease.[161]

T cells are classified based on their function into various categories such as helper T (Th) cells, cytotoxic T (Tc) cells and regulatory T (Treg) cells.

After naïve lymphocytes are activated, they increase in size, proliferate and are known as lymphoblasts. Some of these cells differentiate into effector lymphocytes which have the ability to produce molecules that function in the elimination of antigens. Effector lymphocytes include T helper cells, cytotoxic T lymphocytes (CTLs) and antibody secreting B cells. A state of homeostasis is present when there is a balance between the effector and regulatory T subsets.[137]

T cells are involved in nearly many immunoregulatory interactions both in vivo and in vitro.[136]

T helper cells:

Th cells were so designated because they played a crucial role in the process of antibody production by the B cells.[75]

Parish and Liew (1970) originally described the presence of two different subsets of Th cells-Th1 and Th2. [128]

Mossman and Coffman (1989) first reported that mouse T cell clones can be classified as two functional subsets based on their cytokine profile as type I helper T cells (Th1) and type 2 helper T cells (Th2).[118]

Following infection, the two distinct CD4+ helper T-cell subsets are induced. Their balance determines what kind of adaptive immune response is utilized to eliminate presence of infection.[117]

Th1 cells have been shown to preferentially secrete interleukin-2 (IL-2), interferon- γ (IFN-γ) thus resulting in cell mediated immunity.[36]They increase the ability of macrophages to kill both intracellular and extracellular pathogens. They also function in mediating delayed-type hypersensitivity reactions.[139]

Th2 cells on the other hand secrete IL-4, 5, 6 and 13 and induce humoral immunity that results in B-cell development.[118]

D'Andrea A et al (1993)[43] and **Ohmori et al (1997)[124]** showed that once either Th1 or Th2 cells become dominant they can dampen the development of the other subset, resulting in difficulty to change the immune response pattern.

Zhu J et al (2001)[189] contradicted this by stating that Th2 cells are thought to be a more stable phenotype as opposed to Th1 cells

Th 1/Th2 Paradigm:

Seymour et al (1993)[144] proposed that in non-susceptible individuals, Th1 subsets are found to predominantly lead to cell mediated immunity. IFN-γ expression is up regulated thus producing protective antibodies. On the contrary he found that in individuals susceptible to periodontal breakdown Th2 subsets predominate. Cytokines required for B cell proliferation and differentiation are produced, thereby leading to polyclonal B cell activation and up regulation of nonprotective antibodies along with IL-1 production by B cell.

Ebersole and Taubman (1994)[49] conducted adoptive transfer experiments using the Th2 clone A3. They reported that periodontal disease is abrogated by Th2 cells and Th1 cells aid in disease progression.

Pilon M et al (1991)[131] evaluated the gingival crevicular fluid of periodontitis sites and demonstrated decreased Th1 cytokines.

Fujihashi K et al (1991)[53] reported a decrease in Th1 cytokine levels in gingival mononuclear cells of periodontitis sites.

Sigush B et al (1998)[148] and **Gemmell E (1994)**[60] reported a decrease in Th1 cytokines in the peripheral blood mononuclear cells of periodontitis patients stimulated with mitogens, P.gingivalis and F.nucleatum respectively.

Aoyagi T et al (1995)[4], **Bartova J et al (2000)**[13], **Yamazaki K et al (1994)**[179] reported an increase in Th2 responses in the peripheral blood collected from patients with periodontitis.

Lappin DF et al (2001)[91], **Tokoro Y et al (1997)** and **Yamazaki K et al (1994)** reported an increase in Th2 responses in gingival tissues.

Manhart SS et al (1994)[108] and **Reinhardt RA et al (1989)**[135] reported an increase in Th2 responses in extracted gingival mononuclear cells and gingival crevicular fluid respectively. All these studies support the hypothesis that Th1 cells are associated with a stable lesion and a Th2 response with disease progression.

Ebersole JL et al (1994)[49] **Salvi GE (1998)**[143] **Takeichi O (2000)**[158], on the contrary reported an increase in Th1-type cells and reduced Th2 responses in periodontally diseased tissues indicating that a predominance of Th2 cells denotes a stable lesion and a predominance of Th1 cells denotes a progressive lesion.

Fujihashi K et al (1994)[54] and **Prabhu A et al (1996)[133]** suggested that both Th1 and Th2 cells were involved in sites with periodontitis. This was demonstrated and proved by the presence of mRNA for both Th1 and Th2 cytokines in periodontally diseased tissues.

Factors influencing Th subsets:

Differentiation of Th1 and Th2 T-cell subsets is determined during priming. It is influenced by a number of factors such as

1. Antigen Binding
2. Cytokine environment
3. Antigen presentation
4. T cell activation
5. Costimulatory molecules

Antigen binding:

The antigen diversity of T cells specifically depends on the structural variation of the T cell receptor (TCR). Structurally, TCR consists of two polypeptide chains, α and β or γ and δ chains. **Davis MM and Bjorkman PJ (1988)[46]** demonstrated that the property of antigen recognition by T cells is generated in part by the recombination of germ line V, D, J and C gene segments of TCR

Boyton RJ (2002)[18] studied the process of TCR selection and structure in Th1 and Th2 lines and clones with fixed peptide specificity and class II restriction. The Th2 clones were shown to use TCR complimentary determining region (CDR) 3α loops than their Th1 counterparts. Molecular modeling of Th1- and Th2 – derived TCR showed that Th2 CD3α consisted of larger side chain residues than Th1 TCR. It was also proposed that under Th2 polarizing conditions, there is a trend for CD4+ T cells to have elongated TCR CD3α loops, which are predicted to alter TCR binding and also reduce contact at other interfaces, possibly impeding TCR triggering.

Boyton et al (2002) concluded that either the elongated receptor was lost during selective expansion of Th1 cells or that selection of the Th2 line was compatible with expansion of cells bearing either type of receptor, with the elongated form as the preferred receptor.

Cytokine environment:

Boyton & Altmann (2002)[19] proposed that during Th1 or Th2 development, differential selection from the available pool of specific TCR occurs. They also suggested that local factors like the cytokine or chemokine milieu played an important role in the selection of cells to develop into either Th1 or Th2. They also stated that while many factors can determine the polarization of T cells any single factor can override the changes initiated by the other factors.

Iezzi G (1999) [74] reported that in the presence of IL-12, a short T cell receptor stimulation has been shown to induce Th1 polarization. IL-12 is said to exert its effect during and after TCR signaling. Th2 polarization, on the other hand was shown to require a prolonged TCR signaling and IL-4 was effective only when present during TCR triggering. These authors concluded that duration of TCR stimulation was crucial determinant in influencing Th1/Th2 polarization.

Antigen presentation:

Immune responses may be regulated at the level of antigen presentation. Antigen presenting cells can be classified based on their function as professional and non professional antigen presenting cells.

Haverson et al (1997) and **Taubman MA et al (1997)** demonstrated that antigen presentation by professional antigen-presenting cells (like dendritic cells and macrophages) can result in stimulation of destructive cytokines while presentation by nonprofessional antigen presenting cells (like epithelial or endothelial cells) can result in anergy and no tissue destruction.[69, 160] The mechanism of this anergy is unknown, however it is well established that major histocompatibility complex Class II antigens are expressed by junctional and pocket epithelial cells.[144] Therefore they could be capable of antigen presentation, which could result in anergy and possible apoptosis of the Th1 cells, thereby allowing cells with a Th2 cytokine profile to emerge. Endothelial cells, which in humans constitutively express major

histocompatibility complex class II, could also present antigen leading to anergy.

Antigen presenting cells:

Firstly, the microbe needs to be recognized and captured in the skin/mucosa by APC. To accomplish this they possess a unique array of antigen recognition and capture receptors including the toll-like receptors and C type lectin receptors. The antigen is then processed into smaller peptides. Following which, the peptides are then packed with molecules called major histocompatibility complex (MHC) and presented to the T cells.[146, 169] As mentioned earlier, APCs are further classified as professional APC (dendritic cells, macrophages and B-cells) and non- professional APC (keratinocytes and gingival fibroblasts). The professional APCs express MHC class II molecules and costimulatory molecules.[41, 11]

Dendritic cells:

Steinman and Cohn (1973) described dendritic cells (DCs) as phagocytic cells that have long finger-like processes similar to the dendrites of nerve cells.[153]They are distributed in the lymphoid and the non lymphoid tissues. In the periphery, DCs reside in an immature state with high phagocytic capacity, contributing to their ability to serve as sentinels that survey the tissue for invading microbes. Upon infection, DCs sample these invaders via various mechanisms and initiate a maturation process. The latter involves expression

of chemokine receptor 7 (CCR7) that mediates their migration to the lymph node (LN), and up-regulation of MHC class II and co-stimulatory molecules, which enables potent activation of CD4+ T cells.

Steinman (2007) stated that this capacity of dendritic cells to present antigens via MHC class II (in addition to presentation by MHC class I to CD8+ T cells) awarded DCs with the term professional antigen-presenting cells (APCs). He said that DCs were the most potent APCs activating native T cells, demonstrating their critical function in induction of adaptive immunity.[152]

They occupy a unique niche in the innate immune system by serving as a bridge to the adaptive immune system. They do this by capturing microbes and their antigens while in the immature state and stimulating a T cell response to these antigens in their mature state. They enable in priming naïve helper/cytotoxic T cells to undergo clonal expansion.[12, 67]

To stimulate naive T cells that have never encountered the antigen before, additional signals (i.e. costimulatory signals) which are expressed by dendritic cells are required. Dendritic cells are known to express class II MHC molecules and costimulatory molecules CD80 and CD86. Based on the tissues in which they are present, dendritic cells can be of various types.

Cutler et al (2001) stated that in peripheral tissues of humans, three major types have been described, including two of myeloid origin –

Langerhan's cells and interstitial dendritic cells and the third of lymphoid origin- plasmacytoid dendritic cells.

Kosco-Vilbois MH (**2001)** described that other types include follicular dendritic cells (restricted to primary B-cell follicles) [88]

Caux et al (1997), Jotwani et al (2001), Thomas et al (1993) desribed a class of dendritic cells as those developed from in vitro dendritic cell culture systems (cultured either directly from CD34+ hematopoietic progenitors[29, 30] or precursors differentiated from CD34+ progenitors).[83, 163] CD1a+ Langerhan's cells have been found to increase in number in the epithelium with gingivitis, experimental gingivitis1[122, 170]and periodontitis.[88]It is speculated that these Langerhan's cells are mobilized and matured in response to inflammatory cytokines and pathogen associated molecular patterns from oral mucosal pathogens.[40, 42]

Macrophages:

Macrophages perform the functions of APC only in the presence of infection, where they possess certain types of receptors that recognize differential carbohydrate patterns on foreign cells. They also have receptors for specific bacterial products such as lipopolysaccharide (LPS). When these molecules bind to their bacterial ligands, they stimulate the macrophages to up regulate MHC‑II and B7, providing these cells with strong antigen presentation properties.

Abbas et al (2007) stated that monocytes present in the circulation can migrate to any site of infection or inflammation. They are then capable of transforming into macrophages which phagocytose and kill microbes. Macrophages constitutively express low levels of MHC class II molecules and co stimulators. In the presence of effector Th1 cells (which secrete higher levels of IFN-γ and CD40L) macrophages carry out antigen presentation. CD40 CD40L binding ensures that macrophages present antigen to T cells.[1]

B cells:

Berglundh et al (2007) stated that antigen presentation differs from other professional APC (dendritic cells, macrophages). Antigen is engulfed using an immunoglobulin receptor referred to as the B cell receptor. The antigen is then reduced to peptides and transported to the surface of the B cell for presentation to CD4+ T cells. Binding of CD40 (B cell) to CD154 (activated T cell) is essential also for clonal expansion and co stimulation, apart from interaction of BCR and antigen. [15]

Gingival keratinocytes:

Renne J et al (2010)[138] and **Chung Y et al (2009)**[34] reported the constitutive expression of IL-1α on a keratinocyte. This has been known to promote T cell responses. This is also said to promote autocrine actions, some of which include up regulation of MHC class II expression and stimulation of cytokine and chemokine production.

Sims and Smith (2010) reviewed the function of B cells and stated that it is also said to exhibit juxtacrine actions by activation of neutrophils, monocytes and T cells that accumulate during inflammatory conditions.[149] It has been reported that IL-1α was a general amplifier of T-cell responses in several epithelial tissue immune responses.[27, 121, 173]

Gingival fibroblasts:

Gingival fibroblasts are the most abundant cells of the periodontium and they are known to possess a central role in homeostasis, pathogenesis and healing. These cells are spindle shaped with oval nuclei having one or two minute nucleoli, eosinophilic to basophilic cytoplasm depending on the rate of synthetic activity. They are responsible for intracellular assembly of various extra cellular fibrillar and non fibrillar products such as, procollagen, pro-elastin and glycosaminoglycans.[26, 176]

Danese et al (2004) proposed that fibroblasts could modulate immune cell behavior by conditioning the local cellular and cytokine production. So, activation of fibroblasts would lead to the production of a number of pro-inflammatory cytokines, chemokines and prostanoids such as prostaglandin E2.[44]

Wang Pl and **Ohura K (2002)** added that fibroblasts also expressed adhesion molecules in response to diverse pathogen associated molecular

patterns in periodontal disease including lipopolysaccharide, peptidoglycan and CpG DNA from diverse periodontal pathogens.[174]

Brouty-Boye D et al (2000)[22] and **Hogaboam C.M. et al (1998)**[72] reported that fibroblasts that were obtained from diseased tissues displayed a fundamentally different phenotype when compared to fibroblasts that were taken from normal tissues at the same anatomical site.

The tissue distribution and relative proportion of fibroblast subpopulations have a considerable impact on the regulation of connective tissue function in health and disease.[109]

P.C. Lekic et al proposed that because of the synthetic and remodeling activities of fibroblasts in soft connective tissues, it was evident that the functionally different subpopulations would exert a strong effect on treatment outcomes involving periodontal tissues. As an example, tissue destruction in periodontitis could be improved by methods that directly targeted the extracellular matrix remodeling activities of specific fibroblast subpopulations.[125]

Zhang Y et al, 1998 and Pap T, 2000 suggested that fibroblasts could also regulate the behavior of hematopoietic cells present in damaged tissue via CD40–CD40L interaction, which could lead to the activation of the NF-kB family of receptors. This would cause fibroblasts to synthesize high levels of IL-6, IL-8, cyclooxygenase-2 and hyaluronan.[186, 127] This mechanism is similar

to the crosstalk that occurs between lymphocytes and antigen presenting cells and suggests that it may provide crosstalk between fibroblasts and leukocytes.

T cell activation:

Manhart SS et al (1994) and **Bierer BE (1989)** said that the first step in clonal expansion and activation of antigen-specific T cells is antigen recognition. Peptide antigens are recognized via the TCR. Antigen recognition is the first step in the clonal expansion and activation of antigen-specific T cells. They recognize peptide antigens via the TCR. TCR is expressed as a complex with a CD3 molecule whose δ chains have cytoplasmic tails capable of transmitting an activation signal derived from antigen binding to TCR.[108]CD4 and CD8 molecules act as co receptors with TCR.[16]

Viola A and Lanzavecchia A (1996) said that in order to be recognized by the T cell, the antigen has to be engulfed by APC, processed into antigen peptides and presented on the cell surface by binding to peptide binding groove of MHC. The TCR recognizes and binds to the antigen bound MHC presented by the APC.[146,169] This provides the 1st signal for T cell activation leading to proliferation, differentiation and activation of CD28. The second signal is non-specific in nature and occurs as a result of binding of APC expressed co stimulatory ligands (belonging to the B7 family) to its receptor on the T cell which is an important process for T cell survival and function.

Jenkins MK et al (1991) stated most importantly that, T cells cannot be activated by an antigen signal alone. Without costimulatory signals from costimulatory molecules such as CD28[82,84] and the CD40L,[8] antigen signals make cause T cells to go into a state of anergy rather than activate them. It is now understood that optimal activation of T cells requires both the processes of co stimulation as well as TCR engagement. [51, 66, 86, 32, 57]

Co-stimulation:

The antigenic peptides that are presented by MHC molecules can trigger the T cell receptor (TCR) signaling. However, the T cell function and fate is determined by co-stimulatory and co-inhibitory receptors that are present on the surface of the T cells. These are collectively known as co-signaling receptors.

Bretscher P and Cohn M (1970) reviewed that the discovery of CD28 as a prototype co-stimulatory TCR provided evidence for the two-signal model of T cell activation, according to which both TCR and co-stimulatory signaling are required for full T cell activation.[86, 21, 119] Since then, T cell co-signaling receptors have been broadly defined as cell-surface molecules that can transducer signals into T cells to

a) positively (co-stimulatory receptors) or
b) negatively (co-inhibitory receptors) modulate TCR signaling.

The repertoire of co-signaling receptors expressed on T cells is highly versatile and responsive to changes in the tissue environment. Within a specific tissue environment, the signals that are received from or, sometimes, transduced to the surrounding cells by the given repertoire of T cell co-signaling receptors are determined by the type of ligands or counter-receptors that are expressed on the surface of the cells that interact with T cells.

Liechtenstein T (2012) stated that co-signaling ligands and counter-receptors have been identified on nearly all cell types, although their expression has been most well characterized on professional antigen presenting cells (APCs), as APCs are the primary drivers of T cell activation and differentiation in lymphoid organs.[97]

Most co-signaling molecules are members of the immunoglobulin superfamily (IgSF) and tumor necrosis factor receptor superfamily (TNFRSF). Co-signaling molecules can be further subdivided into specific families on the basis of primary amino acid sequence, protein structure and function. The IgSF includes several co-signaling families.

Greenwald RJ et al (2005) stated that the CD28 and B7 families were perhaps the best-described families of IgSF receptors. The members of the CD28 family were known to interact primarily with members of the B7 family.[63] A costimulatory signal (signal 2) was said to occur through the CD28 molecule, which was recruited to the immunological synapse following TCR

ligation that was provided by B7-1 or B7-2. Like the MHC, the B7 proteins are expressed by APCs.

Powell J et al (1998) stated that he costimulatory signal serves to induce T-cell production of interleukin (IL) -2. IL-2 is known to act in an autocrine/paracrine fashion on the T cells and is obligatory for their survival and differentiation into effector cells.[132] Without the co stimulatory signal, signal 1 from the TCR by itself induces T cells to become tolerant to their cognate antigen instead of activated.[81,59,61] Both the TCR and CD28 are constitutively expressed on most naive T cells, such that the T cell is ready to respond to antigen as presented by an MHC-expressing APC. Cytotoxic T lymphocyte antigen 4 (CTLA-4) functions to inhibit T-cell responses and thus has opposing activities to CD28. Binding of B7-1 or B7-2 with CTLA-4, a homolog of CD28, may inhibit T cell responses by delivering a putative negative signal.[32, 25, 89, 171, 172]

B7 family of molecules:

The B7 family consists of immunomodulatory proteins that are required for the fine tuning of immune responses in addition to the primary signal provided by peptide-MHC complex. They belong to the immunoglobulin (Ig) superfamily of type I transmembrane proteins. Many studies have reported that the co stimulatory interactions that occurred between the B7 family ligands expressed on antigen-presenting cells (APC) and their receptors on T cells were essential for the growth, differentiation,

and death of T cells.[51, 66, 86, 32, 57] The molecules that have been included as members of the B7 family include – B7-1, B7-2, ICOS-L, B7-H1 (PD-L1), B7-DC(PD-L2), B7-H3 and B7-H4.

B7-1 and B7-2:

Freeman GJ et al (1993) suggested that the two distinct CD28 ligands, B7-1 and B7-2, appear to be only distantly related, with an amino acid sequence identity of 26%.[52] B7-1 and B7-2 were thought to exist as monomers and have an extracellular V-like and a C-type domain.

Peach RJ et al (1995) said that conserved residues in B7-1 and B7-2 that were found in these V-and C-like domains have been shown to be critical for the binding activity of B7-1 to CD28 and CTLA-4.[18]

Lenschow DJ et al (1996) reported that the most marked differences between B7-1 and B7-2 were found in the cytoplasmic tail. B7-1 was shown to have a very short cytoplasmic tail, while the cytoplasmic tail of B7-2 was shown to be much longer and also contained three potential sites for phosphorylation by protein kinase C (PKC).[149] However, much of the homology between human and murine B7-1 is concentrated in the Ig-like extracellular domains and not in the cytoplasmic tail. This indicates that B7-2 may not have an important signaling role, but may rather act primarily as a ligand.[84]

June Ch et al (1994) and **Greenfield EA et al (1998)** reported that B7-1 and B7-2 were expressed mainly by professional antigen presenting cells which were in turn activated monocytes, B cells and dendritic cells.[84, 62]

Macrophages are thought to express low levels of CD86. CD80 can be induced after treatment with interferon-γ. CD86 expression is said to be low on B cells until activation is complete, which later induces a rapid up-regulation of these molecules.[80]

Gemmell E et al (2001)[59] reported that the percentage of CD80+ macrophages has been shown to be significantly lower than the percentage of CD86+ B cells and macrophages in gingival tissue sections.

Mahanonda R et al (2002)[106] reported that CD86 was up-regulated mostly on B cells that were isolated from periodontitis lesions. They also showed that a number of periodontopathogenic bacteria including P. gingivalis up-regulated CD86 on B cells in vitro.

Azuma M et al (1993)[9] have reported the expression of B7 molecules by the activated human peripheral blood T cells, CD4 and CD8 clones and natural killer clones.

Hirokawa et al (1995)[70] suggested that B7 molecules were known to function as co-stimulatory molecules on T cells and were thought to aid in clonal expansion of activated T cells.

Another study reported that memory CD4 cells expressed CD86 while naive CD4 cells did not. Naive T cells only expressed CD80 after co stimulation with CD86 and T cell receptor ligation.[65]

B7-H1 (PD-L1) & B7-DC (PD-L2):

The molecules B7-H1[47] and B7-DC[166] are also known as PD-L1 and PD-L2 respectively.

Latchman Y et al (2001) reported that they function as ligands for PD-1, which is an inhibitory receptor on T cells.[92]

According to **Nishimura H and Honjo T (2001)** B7-H1 and B7-DC are potent inhibitory co-stimulatory ligands that have been identified in various tissues like lymphoid, non-lymphoid and tumor cell lines. When these ligands interact with a counter receptor PD-1, they can result in inhibition of T and B cell responses.[123]

The PD-1 receptor is reported to have a role in mediation of immunological self tolerance. The mechanism is still not well understood, but B7-DC is thought to inhibit lymphocyte activation and B7-H1 is thought to co-stimulate lymphocyte function. However, several authors have found that binding of PD-L1 and 2 to PD-1 is costimulatory on T cells.[147, 159, 100]

B7-H2 & ICOS:

B7-H2 is detected on the surface of macrophages and B cells. It functions as a ligand for the inducible costimulatory molecule (ICOS) expressed on antigen-primed T cells. The memory T cells are activated by costimulatory signals via ICOS, with a preference for Th2 responses.[175, 38, 112, 113, 73]

Yoshinaga SK et al (1999)[180] reported its expression by activated and resting memory T cells. Several authors have reported that it is constitutively expressed by B lymphocytes and is induced by the tumor necrosis factor α on non lymphoid cells.[98, 157]

Chapoval AI et al (2001)[33] has demonstrated that the engagement of B7-H2 results in activation of T-helper memory cells with a bias production of Th2 cytokines, such as IL-4 and IL-13.[96]

B7-H3:

The B7-H3 molecule, a homologue of B7 was discovered by **Chapoval AI et al (2001)** in the laboratory of Lieping Chen.[33] Originally, B7-H3 was described as a 2-immunoglobulin like domain. This structure was supported in murine studies done by scientists like **Sun M et al, (2002)**[155] and **Ling V et al (2003)**[91]When studied in a mouse, B7-H3 was shown to be present on chromosome 9 and was said to share an 88% identity and a 93%

similarity with the human B7-H3 molecule. However, human B7-H3 was shown to be encoded on chromosome 15.

Steinberger P et al (2004) reported that the human B7-H3 had a 4 Ig-like structure and that this structure could be due to a duplication of the locus that encoded the B7-H3-IgV and -IgC exons.[151]

Chapoval AI et al (2001), Sun M et al (2002), Zhang GB et al (2005) and **Steinberger P et al, (2004)** have reported that its expression is induced in T cells, B cells, monocytes, dendritic cells (DCs) and some tumor cell lines.[185] B7-H3 is also reported to be found on the surface of non-professional APCs including fibroblasts, fibroblast-like synoviocytes, and epithelial cells.

Hofmeyer KA et al (2008)[71] reported that B7-H3 is found in most of the organs at the transcriptional level. At the protein level, this molecule is reported to be found in the human liver, lung, bladder, testis, prostate, breast, placenta, and lymphoid organs. They also suggested that due to its posttranscriptional regulation different expression patterns have been observed between B7-H3 mRNA and protein.

Zhang G et al (2008)[183] demonstrated that human soluble B7H3 (sB7-H3) was cleaved by MMPs from the surface of activated T cells, monocytes and monocytes-derived dendritic cells which were in turn able to bind its receptor on activated T cells.

Zhang G et al (2010)[184] reported that the process of binding to co stimulatory receptors on monocytes /macrophages by sB7-H3 results in the secretion of proinflammatory cytokines.

Zang X et al (2003)[181] and **Hashiguichi et al (2008)**[68] demonstrated that the triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2, or TREML2), is the co stimulatory receptor for B7H3 particularly in CD8 T cells.

There has been however, a contradictory concept demonstrated by **Leitner et al (2009)**[94] where they showed that B7-H3 was not a co stimulatory ligand for TREML-2 receptor. This was supported by several authors who reported that B7-H3 was not a co-stimulatory molecule but an inhibitory molecule. This function has been reported to be mediated by its binding to an unknown receptor as reported by **Hofmeyer KA et al (2008)**

Cell culture studies:

B7-H3 as a co inhibitor:

Steinberger P et al (2004)[151] demonstrated the expression of B7H3 by both immature and mature monocyte derived dendritic cells. They studied its binding to an inhibitory receptor on T cells as stated that its binding does not stimulate T cell proliferation.

Mahnke et al (2007)[107] showed that when there was a contact between dendritic cells (DC) and CD4+CD25+regulatory T cells (Tregs) there was an up regulation of B7-H3 molecule on DC surface. They also demonstrated that the number of MHC-peptide complexes were decreased.

Murine studies:

B7-H3 as a co stimulator:

Chapoval AI (2001)[33] reported that B7-H3 Ig fusion protein increases CD4+ and CD8+ T cells and selectively stimulates IFN-γ production.

Hashiguichi M et al (2008)[68] demonstrated that the binding of B7-H3 to TLT-2 resulted in the proliferation of CD8+ T cells and up regulation of IFN-γ production by CD8+ T cells.

B7-H3 as a co inhibitor:

Prasad DV et al (2004)[134] demonstrated the expression of B7-H3 by all professional APCs and a minor subset of CD4+ and CD8+ T cells. It was found to inhibit transcriptional factors like AP 1, NFAT and NF-κB that regulate T cell activities. Murine B7-H3 was also shown to inhibit T-cell activation and effector cytokine production.

Human studies:

B7-H3 as a co inhibitor:

Ling V et al (2003)[99] demonstrated the role of B7-H3 in the down regulation of proliferation of CD4+ T cells and also the effector cytokine production.

Expression of B7-H3 in systemic diseases:

Animal studies

B7-H3 co inhibitor:

Suh WK et al (2003)[154] reported that mice deficient in B7-H3 developed a more severe airway inflammation compared to those that expressed the same. They also added that the lesions had predominantly Th1 cells. They reported that B7-H3 is up regulated in the presence of IFN-γ and down regulated in the presence of IL-4.

Prasad DV et al (2004)[134] showed that on exposure to autoimmune encephalomyelitis in B7-H3 deficient mice, the condition was exacerbated.

Human studies:

B7-H3 as a co stimulator:

Kim J et al (2005)[87] stated that B7-H3 was the most abundant co stimulatory molecule detected by flow cytometry on cultured respiratory tract

epithelial cells. Engagement of B7-H3 ligands were said to result in the proliferation of CD4+ and CD8+T cells, a bias toward Th1 cytokine production, and primary cytotoxic T cell activation. They concluded that the presence of B7 homolog on epithelial cells may play a role in driving expression of the Th1 and Th2 cytokines observed in asthma and CRS.

B7-H3 as a co inhibitor:

Tran et al (2008)[165] reported that expression of B7-H3 was associated with FLS rich areas and was in close proximity to T cells in the RA pannus. The expression of B7-H3 was found to be constitutive and uninfluenced by immunoregulatory cytokines. They also suggested that B7-H3 could be an important signaling molecule between FLS and T cells. They reported that B7-H3 was found to bind to inhibitory receptor on unstimulated T cells and activating receptor on cytotoxic T cells.

Expression of B7-H3 in cancer immunity:

In vitro studies:

B7-H3 as a co stimulator:

In the laboratory, studies have been carried out with some commonly used human cancer lines like HL-60 promyelocytic leukemia, K562 myelogenous leukemia, SW480 colon adenocarcinoma, A549 epithelial lung

adenocarcinoma, and G361 melanoma. They were found to express increased levels of B7-H3 mRNA.[33]

Animal Studies:

B7-H3 as a co inhibitor:

Sun X et al (2003)[156], **Luo L et al (2004)[103]**, **Luo L et al (2006)[104]** and **Lupu CM et al (2006)[105]** reported that on studies done on mouse cancer models, an ectopic expression of B7H3 lead to activation of tumor-specific CTLs that were able to slow, tumor growth if not even completely eradicate them.

Human studies:

B7-H3 as a co stimulator:

B7-H3 is found to be expressed by many human cancers. Numerous studies suggest that prostate cancer, non-small-cell lung cancer, gastric carcinoma, ovarian cancer, renal cell carcinoma, urothelial cell carcinoma, and neuroblastoma, also express B7-H3.

Castriconi R et al (2004)[28] reported that 4Ig B7-H3 protects tumor cells in bone marrow (especially neuroblastoma) from natural killer cell (NK cells) mediated killing. They also demonstrated that the binding of 4IgB7-H3 to its receptor on NK cells resulted in inhibition of its actions.

Crispen PL et al (2008)[39] showed that B7-H3 expression by either clear cell renal cell carcinoma (RCC) or the tumor vasculature was found to be significantly associated with an increased risk of death from RCC. Similarly, a marked increase in B7-H3 expression was observed in majority of prostate cancers, and statistically correlated with poor prognosis.[140]

It was also noted that in the case of urothelial cell carcinoma, [17]non small-cell lung cancer,[178] and pancreatic cancer immunohistological studies show that increase in B7-H3 expression correlated with cancer progression.

B7-H3 as a co inhibitor:

Studies done on gastric carcinoma have been shown to correlate with increased levels of B7-H3 expression by cancer with an increased survival rate in patients.

Loos et al (2009)[102] examined the expression of B7-H3 by immunohistochemistry in pancreatic cancer tissue specimens from 68 patients who underwent surgical resection. They reported that the elevated expression of B7-H3 by tumor cells in pancreatic cancer was found to be associated with an increased expression of cytotoxic T cells that correlated with the anti-tumor response.

To summarize, B7-H3 has been speculated to have a co-stimulatory role by some authors and a co-inhibitory role by others.

B7-H3 AS A CO-STIMULATOR	**B7-H3 AS A CO-INHIBITOR**
1) Chapoval AI, 2001[33] 2) Hashiguichi M et al, 2008[68] 3) Kim J et al, 2005[87] 4) Castriconi R et al, 2004[28] 5) Crispen PL et al, 2008[39]	1) Steenberger JP et al, 2004[151] 2) Mahnke et al, 2007[107] 3) Prasad DV et al, 2004[134] 4) Ling V et al, 2003[99] 5) Suh WK et al, 2003[154] 6) Tran et al, 2008 [165] 7) Sun X et al, 2003[156] 8) Luo L et al, 2004[103] 9) Lupu CM et al, 2006[105] 10) Loos et al, 2009[200]

Materials and Methods

MATERIALS AND METHODS

Gingival fibroblasts were cultured from tissues obtained during crown lengthening and were evaluated for expression of B7-H3 by immunocytochemistry. The results obtained were confirmed by flowcytometry.

STUDY POPULATION:

The study population comprised of patients selected randomly from the Department of Periodontics, Ragas Dental College and Hospital, Chennai. The Institutional Review Board of Ragas Dental College and Hospital approved the study protocol. After explaining the study protocol to the patients, an informed consent was obtained prior to conducting the study. A total of 7 periodontally healthy patients ranging from 20-50 years of age were selected for the study.

INCLUSION CRITERIA:

Patients who were referred to the Department of Periodontics for crown lengthening by gingivectomy procedure were selected for the study. The site characteristics include

1. Absence of bleeding on probing
2. Probing depth (PD) $\leq$ 3mm
3. No evidence of attachment loss

EXCLUSION CRITERIA:

1. Patients with a history of antimicrobial therapy for the past 3 months
2. Patients who had undergone periodontal therapy over the previous 6 months
3. Patients who were smokers
4. Patients with systemic disorders and other dental problems (like pulpal disease)

GINGIVAL TISSUE SAMPLE COLLECTION:

Gingival tissues were obtained after making an external bevel incision in the site that required crown lengthening in healthy individuals who were referred to the Department of Periodontics, Ragas Dental College and Hospital, Chennai.

TRANSPORTATION OF TISSUE TO LABORATORY FOR CULTURE:

Dulbecco's Modified Eagle Medium (DMEM) without serum at a pH of 7.2-7.4 with 5 times (5x) the concentration of antibiotics (Penicillin-100 IU, Streptomycin-100µg/ml, Amphotericin B-1µg/ml, maintained at 4°C with an ice pack) was used to transport tissue specimens. They were transported in leak proof sterilized culture vials.

PROTOCOL FOR WASHING THE TISSUE:

1. First, the media was discarded from the plate
2. Residual serum was removed by washing twice with 2ml D-PBS
3. 1ml trypsin 0.25% with EDTA 0.05% was added to the 60 mm plate
4. The plate was left stationary for 1 minute inside the incubator and it was made sure that the monolayer was completely covered
5. The tissue specimen obtained was washed with sterile D- Phosphatated Buffered Saline (PBS) containing 5 times (5x) antibiotics (Penicillin-100 IU, Streptomycin - 100µg/ml and Amphotericin-1µg/ml), in order to remove tissue debris and blood clots, under the sterile environment of a laminar hood

PROTOCOL FOR PRIMARY CULTURE:

1. A No-15 scalpel was used to mince the tissue finely into about 1mm size pieces
2. The minced pieces were then incubated with crude collagenase in working media for 18 hours (3mg/ml) at 37°C in an atmosphere of 10% CO_2 and 90% air
3. The supernatant was removed by centrifuging at 2400 rpm for 5 minutes

4. Fresh working medium was added to the sediment and plated on a 60mm plate
5. Supernatant obtained was again centrifuged at 2400 rpm for 5 minutes and if sediment was obtained, it was plated on a 60mm petri plate
6. A humidified atmosphere of 10% CO_2 and 90% air was used to maintain the culture
7. After a period of 48 hours, the attachment of cells was observed
8. Change of media was performed every third day
9. Within a period of 2-3 weeks, a monolayer of cells was formed

PROTOCOL FOR SUBCULTURE:

1. The culture was carefully examined to rule out any signs of deterioration or contamination
2. To check whether the cells were rounding up, the monolayer was examined under a microscope
3. The plate was then tapped at the bottom until all the cells were detached. It was noticed that when the plate was tilted, cells slid down the surface
4. Cells suspended in trypsin were collected in a centrifuge tube and centrifuged at 2400 rpm for 3 minutes
5. After centrifugation, the supernatant was discarded. The medium was added to the remaining cell plate and pipetting was repeated to disperse the cells

6. The cells were then counted with a haemocytometer
7. The cell suspension was diluted by appropriate seeding concentration by adding adequate volume of cell to a pre- measured volume of medium in a culture plate
8. After the plates were closed, they were returned to the incubator

CELL CULTURE STUDIES:

Observation of the f_1, f_2, f_3 fibroblast sub population in cell lines

1. All cell lines from the fifth passage were placed on three 60mm tissue culture plates at a concentration of $0.5x10^4$ cells/ml
2. Using a phase contrast microscope at 20x magnification, cells were observed and counted for 8 consecutive days and classified according to their morphology into f_1, f_2 and f_3 fibroblasts according to the description by **Mollenhauer and Bayreuther (1986)**[114]
3. 30 cells were randomly recorded in each tissue culture plate giving a total of 90 cells per cell line

Observation and recording of the mitotic and post mitotic fibroblast sub population in cell lines

1. All cell lines from the fifth passage were placed on three 60 mm tissue culture plates at a concentration of $0.5x10^4$ cells/ml
2. Cells were observed and counted for 8 consecutive days and classified according to their morphology into mitotic (f_1, f_2, f_3) and post mitotic

(f_4, f_5, f_6, f_7) fibroblasts according to the description by **Bayreuther et al (1988)**[14] This was done using a phase contrast microscope

3. 30 cells were observed randomly and recorded in each tissue culture plate giving a total of 90 cells per cell line

Evaluation of growth curves and its derivatives in fibroblast cell lines

1. From each culture, cells were inoculated at $1.2x10^4$ cells/ml/well on 24 welled plates
2. There was an overnight attachment that occurred, after which cells from 3 randomly selected wells were trypsinized. They were then counted using a haemocytometer
3. Change of medium was done on the 3^{rd} and 6^{th} days
4. The count should was repeated every 24 hours for 8 days
5. To avoid the occurrence of any error, cells were counted thrice
6. To plot the growth curve, the average of daily cell counts of each well was calculated
7. To estimate the seeding efficiency, the seeding cell count and the cell count on the first day i.e.) 12 hours of seeding was used. This was expressed in percentage by the equation:

$$\frac{\text{Cell count/well/ml after 12 hours x 60}}{\text{Seeding cell count/well/ml}}$$

8. At its log phase, the population doubling time was calculated from the slope of the growth curve

Fibroblasts were identified based on their cell morphology when they were examined under the microscope. The fibroblasts were confirmed to be gingival fibroblasts based on the expression of vimentin and CD40 which has been documented to be a marker of gingival fibroblasts. The protocol for the staining of vimentin and CD40 is given in **Annexure 2**

IMMUNOCYTOCHEMISTRY B7-H3:

Cells were fixed on APES coated slides using methanol, par formaldehyde, acetone and immunologically stained for B7-H3 (CD276)

APES COATING PROCEDURE:

The slides were first allowed to cool down and then coated with APES using the following steps:

1. Slides were immersed in a Couplin jar with acetone for 2 minutes
2. The slides were then immersed in a Couplin jar containing APES for 5 minutes
3. After this, the slides were immersed in two changes of distal water for 2 minutes each, to remove excess APES. They were then allowed to dry.

REAGENTS USED:

1. Distilled water
2. Acetone

3. Xylene
4. Absolute alcohol
5. Alcohol 70%
6. Alcohol 50%
7. Hydrogen peroxide 3%
8. EDTA buffer (Ph 8.0)

ANTIBODIES USED:

Primary Antibody:

R&D systems, Human B7-H3 Biotinylated Affinity Purified Polyclonal Antibody (Goat IgG 50µg), Catalog Number BAF 1027, Minneapolis, USA

Immunocytochemistry kit:

R&D systems Anti-Goat HRP-DAB cell and tissue staining kit, Catalog Number CTS008, Minneapolis, USA

- Peroxidase blocking reagent – 6mL of 3% Hydrogen peroxide (H_2O_2)
- Avidin blocking reagent – 6mL Avidin solution containing 0.1% sodium azide (NaN_3)
- Biotin blocking reagent – 6mL of Biotin solution, containing 0.1% NaN_3

- "Vial A" Secondary Biotinylated antibodies-6mL of either anti-mouse, anti-rabbit, anti-goat, anti-rat or anti-sheep secondary antibodies, respectively in 0.01M PBS containing 0.1% NaN_3
- "Vial B" High sensitivity Streptavidin HRP Conjugate (HSS-HRP)-6mL of Streptavidin conjugated to HRP in 0.01M PBS containing 1% carrier protein with preservatives and stabilizer
- DAB chromogen- 2mL of 2.5% 3,3-diaminobenzidine (DAB) in stabilizing buffer
- DAB chromogen buffer – 2 vials (15mL/vial) of 0.1% H_2O_2 in Tris Hcl buffer

EQUIPMENTS:

1. Glass slides
2. Micro centrifuge tubes [Tarsons ^{TM}]
3. Couplin jars
4. Cryoboxes [Tarsons ^{TM}]
5. Humidifier chamber
6. Electronic timer
7. Cover slips
8. Light microscope

Growing cells on a slide:

1. Cell culture was transferred to the wells of a chamber slide or slides
2. Cells were allowed to reach confluence with the addition of fresh media
3. Cells were thoroughly washed in PBS for 5x2 minutes

Fixation:

1. Cells were fixed with methanol/acetone at -20°C for a period of 5-10 minutes
2. Rinsing was done for 3x5 minutes in PBS

Endogenous Peroxidase Blocking:

1. Endogenous peroxidase was blocked by incubating in 0.1-1% H_2O_2 in PBS for 5-10 minutes
2. Rinsing was done for 3x5 minutes in PBS

Blocking of non specific binding:

1. Blocking was done with 1.5% normal serum in PBS for 1 hour
2. The normal serum was from the same species in which the secondary antibody was raised. (Alternatively, 5% BSA could sometimes be used as a blocking agent

Primary Antibody Incubation:

1. The primary antibody was diluted to the recommended concentration in 1.5% normal blocking serum and PBS
2. The Blocking buffer was removed from the slides
3. Primary antibody was added to each slide. Incubation was done at room temperature for 30 minutes or overnight at 4°C.
4. The primary antibody solution was removed and slides were rinsed for 3x5 minutes in PBS

Secondary Antibody Incubation:

1. The Biotinylated secondary antibody was diluted in normal blocking serum and PBS
2. The excess fluid was removed from the slide and the secondary antibody solution was added into each slide. Incubation was carried out at room temperature for 30 minutes.
3. Rinsing was done for 3x5 minutes in PBS and excess fluid was removed

Color Development:

1. AB enzyme reagent was added to each slide. Incubation was done at room temperature for 30 minutes
2. It was then washed for 3x5 minutes in PBS on an orbital shaker. Excess fluid was removed

3. To each well, 1-3 drops of peroxidase substrate was added. Incubation was done at room temperature for 30 seconds-10 minutes.
4. Once the cells started turning brown (can be observed under a microscope), they were washed for 2x5 minutes in PBS on the shaker

Counter stain:

1. The slides were dipped into a staining dish containing hematoxylin for 5-10 seconds
2. Immediately they were washed with several changes of de ionized water
3. They were then removed and placed into an acid bath (200ml dH_2O and 1-3 drops of acetic acid
4. Finally, rinsing was done with distilled water.

Cover slips:

A cover slip was added to the slide for examination under a microscope

IMMUNOCYTOCHEMISTRY PROTOCOL FOR B7-H3:

Wash in PBS for 5x2 minutes

↓

Fix with methanol/acetone for 5-10 minutes

↓

Wash in PBS for 3x5 minutes

↓

Cover with 1-3 drops of peroxidase blocking reagent for 5 minutes

↓

Rinse and wash in buffer for 5 minutes

↓

Incubate with 1-3 drops of serum blocking reagent for 15 minutes

↓

Incubate with 1-3 drops of Avidin blocking reagent for 15 minutes

↓

Rinse with buffer and drain slides

↓

Incubate with 1-3 drops of Biotin blocking reagent for 15 minutes

↓

Rinse with buffer and drain slides

↓

Incubate with primary antibody

↓

Rinse with buffer for 3x15 minutes

↓

Drain slides

↓

Incubate with 1-3 drops of Biotinylated secondary antibody for 30-60 minutes

↓

Rinse with buffer for 3x15 minutes

↓

Drain slides

↓

Incubate sample with 1-3 drops of HSS-HRP for 30 minutes

↓

Rinse with buffer for 3x2 minutes

↓

Drain slides

↓

Calculate the total volume of DAB chromogen needed for the entire reaction

↓

Mix DAB chromogen and DAB chromogen buffer in a dropper bottle

↓

Add 1-5 drops of DAB chromogen solution and incubate for 3-20 minutes

↓

Monitor intensity of staining

↓

Rinse with distilled water for 5 minutes

↓

Counter stain with hematoxylin

↓

Drain excess mounting medium and dry

↓

Slides are ready for observation under a microscope

POSITIVE CONTROL:

Prostate cancer paraffin embedded tissue sections were used as a positive control **(Fig 4 A)**

NEGATIVE CONTROL:

Prostate cancer paraffin embedded tissue sections to which the primary antibody was not added were used as a negative control **(Fig 4 B)**

FLOWCYTOMETRY:

The flowcytometry was carried out at the Centre for Stem Cell Research (CSCR), Christian Medical College, Vellore.

The presence of B7-H3 (CD276) in gingival fibroblasts was analyzed using flowcytometry.

A part of the tissue (3x2mm) collected during gingivectomy for the purpose of crown lengthening was placed in a nutritional medium (Dulbecco's modified eagle medium, (DMEM) containing 10% fetal calf serum (FCS), and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml, and Amphotericin B 100 µg/ml) and taken to the cell culture laboratory.

Characterization - Flowcytometry:

The cells after trypsinization were equally aliquoted, that is 100000 cells per reaction were used, into FACS tubes and stained on live cells with respective antibody. Unstained antibody and cells stained with isotype antibody acted as controls. To avoid bleaching, antibodies were added to the cells in dark. After antibody addition to the samples, they were incubated in dark at room temperature for 20 minutes. 1 ml of PBS, without calcium and magnesium (Lonza) was used to wash the cells and they were centrifuged at 300g for 5 minutes. The pelleted cells were re-suspended in 300µl DPBS, without calcium and magnesium and analyzed with flow cytometer (FACS Calibur; Becton and Dickinson, USA). A minimum of 9000 events were acquired from each sample for analysis using cell quest (BD cell quest pro software, version 5.1 USA).

FACS analysis – B7-H3 (CD276)

Cells after trypsinization were equally aliquoted (1x10^5 cells per reaction) into a facs tube and stained on live cells with Anti-Human B7 –H3 **(**CD 276) (Biotinylated Goat IgG -primary antibody). Unstained cells acted as controls. Antibodies were added to the cells in dark to avoid bleaching. After addition of the antibody the sample was incubated at room temperature in dark for 20 minutes. Cells were washed with 1ml of DPBS without calcium and magnesium and centrifuged at 300g for 5 min. The pelleted cells were resuspended in 100µl DPBS w/o calcium and magnesium and Secondary

Antibody (Anti-Goat; Streptavidin APC-Cy7) was added and incubated in dark at room temperature. A DPBS was performed and cells pelleted and resuspended in 400ul of DPBS and analyzed with a flowcytometer (FACS Calibur; Becton Dickinson). A minimum of 10^4 gated events were acquired from each sample for analysis using cell quest.

Antibodies:

The cells were tagged with Fluorescein Iso Thio Cyanate (FITC), Phycoerythrin (PE) and Allophycocyanin (APC) labeled antibodies. The labeled cells were characterized by FACS ARIA III (BD-Biosciences). Mouse IgG1 was used as an isotype control with the above mentioned dyes.

FITC labelled surface markers are CD29, CD 105, CD14, CD34, CD45 and HLA-DR.

CD34, CD29 and CD105 were used to characterize the gingival fibroblasts. CD14, CD45 and HLA-DR were evaluated to see if gingival fibroblasts expressed immune molecules other than B7-H3

Information on Primary and secondary antibodies used for flowcytometry are provided in the table below:

ANTIBODIES	CONJUGATE	CAT NO	COMPANY
Anti- human CD14	FITC	11-0149	e Bioscience
Anti –human CD34	FITC	11-0349	e Bioscience
Anti –human CD45	FITC	11-9459	e Bioscience
Anti –human CD29	FITC	11-0299	e Bioscience
Anti –human CD105	APC	17-1057	e Bioscience
Mouse IgG 1 K isotype control	FITC	11-4714	e Bioscience
Mouse IgG 1 K isotype control	PE	12-4714	e Bioscience
Mouse IgG I K isotype control	APC	17-4714	e Bioscience
Anti-Human CD 276 (hB7-H3) Goat IgG	biotinylated	BAF1027	R&D Systems
Streptavidin APC-Cy7	APC-Cy7	554063	BD-Pharmingen

STATISTICAL ANALYSIS

Data entry and descriptive analysis was performed using SPSS version 10.0.5. Mean LI was calculated to assess B7-H3 expression.

The intensity of staining was determined by two examiners and the inter examiner variation was determined by kappa analysis.

The mean value and standard deviation for expression of B7-H3 in gingival fibroblasts by flowcytometry was calculated using SPSS version 10.05.

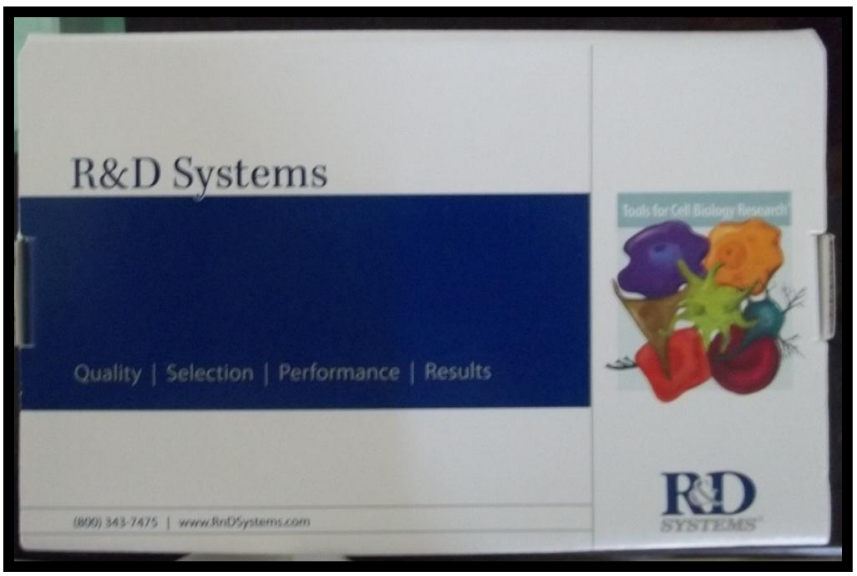
Photographs

ARMAMENTARIUM

Immunocytochemistry:

COUPLIN JARS

R & D KIT – B7-H3



REAGENTS


Serum Blocking
Part No. 865015
FOR RESEARCH USE
Avidin Block
Part No. 865
FOR RESEARCH
Biotin Bloc
Part No. 86
FOR RESEARCH
R&D SYSTEMS
Minneapolis, MN
(800) 343-7475
Made in the USA


DAB Chromogen Buffe
Part No. 860005
FOR RESEARCH USE ONLY
DAB Chrom
Part No. 860
FOR RESEARCH
R&D SYSTEMS
Minneapolis, MN 55413
(800) 343-7475
Made in the USA


Biotinylated
Anti-goat Antibo
Part No. 865003
FOR RESEARCH USE
HSS-HRP
Part No. 865006
FOR RESEARCH USE
R&D SYSTEMS
(800) 343-7475
Made in the USA

Flowcytometry:

BD FACS CALIBUR FLOWCYTOMETER

INVERTED PHASE CONTRAST MICROSCOPE

NORMOXIA 5% CO_2 INCUBATOR

THERMO SCIENTIFIC LEVEL 2 BIOSAFETY

37° WATERBATH

Results

RESULTS

A total of 7 healthy gingival samples were evaluated for expression of B7-H3 in fibroblast cells. Gingival fibroblasts were cultured in complete media from the marginal gingiva of healthy subjects. On reaching confluence, they were trypsinized and split into two plates. Fibroblasts used in this experiment were from the fifth passage.

Of all the 7 samples collected, only 5 gingival fibroblast populations could be cultured successfully.

The duration for enzymatic degradation was 18 hours for each sample respectively. On observing the disaggregated gingival tissue 48 hours after plating, cluster of cells were seen arising from each focus of cells or single cells scattered in the plate. **(Fig 1)**

The size of the colony was calculated depending on the number of cells. (Up to 10 cells were considered as a small colony and more than 10 cells were considered as a larger colony). The plates were observed everyday till confluence was reached. **(Fig 2)** Media change was done every third day. The primary culture reached confluence (70-80%) in approximately 30-35 days. The first subculture derived cells took about 10 days to reach confluence. The second subculture derived cells took about 4-6 days to reach confluence. The third subculture derived cells took about 6-9 days to reach confluence. The fourth subculture derived cells took about 7-10 days to reach confluence. Cells

from the fifth passage were used to assess the growth characteristics and sub population analysis. Growth characteristics and morphology were assessed for all the 5 successful samples. The details of the successful samples are given below.

Culture characteristics:

Cells in the primary culture appeared to grow outwards from a central bit of tissue (**Fig 3)** found in 3-4 regions of the culture plate. Cell growth was satisfactory and cells took about 28-33 days to reach 70% confluence for passaging. Cells from passage 5 were grown on slides for immunocytochemical analysis in 80mm culture plates and the remaining cells were used to study the growth and phenotypic characteristics.

Growth curve and its derivatives: (Graph 1 and Table 1)

The initial seeding concentration was $1.2x10^4$cells/well/ml. The seeding efficiency ranged from was 52.87% to 80.37%. There was a steady increase in the slope of the growth curve from Day 1 to Day 5. The population doubling time was found to range from 24.48 hours to 49.82 hours.

f1:f2:f3 Fibroblast sub population ratio: (Graph 2, 3 and Table 2, 3)

According to Mollenhauer et al, 1986, f1, f2 and f3 proportions were analyzed. There was an increase in f2 and f3 sub populations of fibroblasts and a decrease in f1 sub population of fibroblasts over an 8 day observation period.

Mitotic and post mitotic fibroblast subpopulation ratio: (Graph 4, 5, 6 and Table 4, 5, 6, 7)

According to **Klaus Bayreuther et al (1988)** sub types f1, f2 and f3 were together considered as mitotic and f4, f5, f6 and f7 together constituted the post mitotic sub populations. There existed a negative correlation between the mitotic and post mitotic sub population and that was statistically significant ($p < 0.05$)

Although based on the morphology and growth characteristics of the cells, the cells were identified to be fibroblasts, confirmation was done through the presence of vimentin and CD40

All these cells stained positive for Vimentin - Intense staining (Grade 3) in Passage 5. Vimentin is known to be a marker for fibroblasts and therefore all the cells cultured in our study were confirmed to be fibroblasts. **(Graph 7 and Table 8)**

Intense staining (Grade 3) with CD40 was seen in passage 5. CD40 is known to be a marker for gingival fibroblast and therefore the cells in our study were confirmed to be gingival fibroblasts. **(Graph 8 and Table 9)**

Immunocytochemistry: (Graph 9, 10 and Table 10, 11)

B7H3(CD 276) – All the specimens examined stained positively for B7-H3. The staining was observed both in the cell surface and cytoplasm. There was no staining present in the nucleus of the cell. **(Fig 5, 6)**

Moderate staining was observed in 4 of the 5 samples while intense staining was observed in 1, with a mean labeling index of 47.46 The slides were observed by two examiners and the results are shown in **Table 12.**

When the inter- examiner variability was assessed using the kappa analysis, a good inter-examiner agreement was observed (0.720).

Flowcytometry:

Phenotypic characterization of gingival fibroblasts by flowcytometry (BD FACS ARIA III)

The cells after trypsinization were equally aliquoted, that is 100000 cells per reaction were used, into FACS tubes and stained on live cells with respective antibody. Unstained antibody and cells stained with isotype antibody acted as controls. The pelleted cells were re-suspended and analyzed with a flow cytometer (FACS Calibur; Becton and Dickinson, USA). A minimum of 9000 events were acquired from each sample for analysis using cell quest (BD cell quest pro software, version 5.1 USA).

- Unstained **(Graph 11)**
- Negative control – IgG-FITC **(Graph 12)**

The gingival fibroblasts were found to express

- CD29 (100%) **(Graph 13)**
- CD105 (26.6%) **(Graph 14)**

The gingival fibroblasts did not express

- CD14 (1.45%) **(Graph 15)**
- CD34 (0.62%) **(Graph 16)**
- CD45 (0.36%) **(Graph 17)**
- HLA-DR (0.11%) **(Graph 18)**

FACS analysis - B7-H3 (CD276)

Cells after trypsinization were equally aliquoted (1x10^5 cells per reaction) into a Facs tube and stained on live cells with Anti-Human CD 276 (Biotinylated Goat IgG -primary antibody). Unstained cells acted as controls. Antibodies were added to the cells in dark to avoid bleaching. After addition of the antibody the sample was incubated at room temperature in dark for 20 minutes. Cells were washed and the pelleted cells were re-suspended. A minimum of 10^4 gated events were acquired from each sample for analysis using cell quest.

The results observed were as follows:

The gingival fibroblasts expressed **99.3%** positive staining for B7-H3. **(Graph 19, Table 13)**

Tables and Graphs

Table 1: GROWTH CURVE DERIVATIVES

Days	Cell/well/ml x10^4	Slope	S.E	Population doubling time(hours)	Seeding efficiency%
0	1.2	0.6403	0.078	24.48	66.75
1	0.801				
2	1.58				
3	1.79				
4	2.48				
5	2.86				
6	3.79				
7	4.95				
8	6.32				

S.E-Standard error

Table 2: SUB POPULATION PROPORTIONS IN 90 CELLS FOR 8 DAYS

Days	f1	f2	f3
1	76	11	3
2	70	16	4
3	62	21	7
4	53	27	10
5	52	28	10
6	60	24	6
7	55	26	9
8	48	30	12

Table 3: CORRELATION COEFFICIENT OF THE SUB-POPULATIONS

Subpopulation	Correlation coefficient(r)	P value
f1	-0.848	0.008*
f2	0.864	0.006*
f3	0.785	0.021*

*- ($p<0.05$) statistically significant

Table 4: f1 to f7 SUB – POPULATION PROPORTIONS IN 90 CELLS FOR 8 DAYS

Days	f1	f2	f3	f4	f5	f6	f7
1	66	19	5	0	0	0	0
2	65	17	8	0	0	0	0
3	50	21	19	0	0	0	0
4	54	16	16	2	1	1	0
5	62	14	11	1	1	1	0
6	44	22	24	0	0	1	0
7	42	29	18	0	0	0	1
8	40	22	23	3	0	0	2

Table 5: MITOTIC AND POST MITOTIC POPULATION PROPORTION

Days	Mitotic	Post mitotic
1	90	0
2	90	0
3	90	0
4	86	4
5	87	3
6	89	1
7	89	1
8	85	5

Table 6: CORRELATION COEFFICIENT BETWEEN DAYS AND FIBROBLAST SUBPOPULATION

Subpopulation	Mitotic	Post-mitotic	P value
Correlation coefficient(r)	0.618	-0.618	0.103

*- ($p<0.05$)statistically significant

Table 7: CORRELATION COEFFICIENT BETWEEN MITOTIC AND POST-MITOTIC POPULATION

Subpopulation	Correlation coefficient(r)	P-value
Mitotic	-1.00	.000*
Post-mitotic		

*- ($p<0.05$)statistically significant

Table 8: VIMENTIN – STAINING INTENSITY GRADES

Staining intensity - Grades

Sample	Passage 5
Staining intensity	3

0- no staining

1- Mild staining

2- Moderate staining

3- Intense staining

Table 9: CD40- STAINING INTENSITY GRADES

Staining intensity- Grades

Sample	Passage 5 (1*Ab -30 minutes)	Passage 5(1*Ab- overnight)
Staining intensity	1	3

0- no staining

1- Mild staining

2- Moderate staining

3- Intense staining

Table 10: EXPRESSION OF B7-H 3 USING IMMUNOCYTOCHEMISTRY

Healthy gingival sample	Passage 5(without blocking serum)
Staining intensity	2

0- no staining

1- Mild staining

2- Moderate staining

3- Intense staining

Table 11: STAINING INTENSITY – GRADES (B7-H3)

Healthy gingival sample	Passage 5 (1*Ab -30 minutes)	Passage 5(1*Ab-overnight)
Staining intensity	1	2

0- no staining

1- Mild staining

2- Moderate staining

3- Intense staining

Table 12: INTER EXAMINER VARIABILITY AND LABELING INDEX

S NO.	AGE/SEX	EXAMINER 1	EXAMINER 2	LI	SD
1	42/F	++	++	44.7	**18.8887**
2	30/F	+++	+++	29.8	
3	25/M	++	++	56.1	
4	35/F	++	++	75.3	
5	22/M	++	++	31.4	
			MEAN	**47.46**	

***Clinical Attachment loss (CAL), Labeling index (LI)**

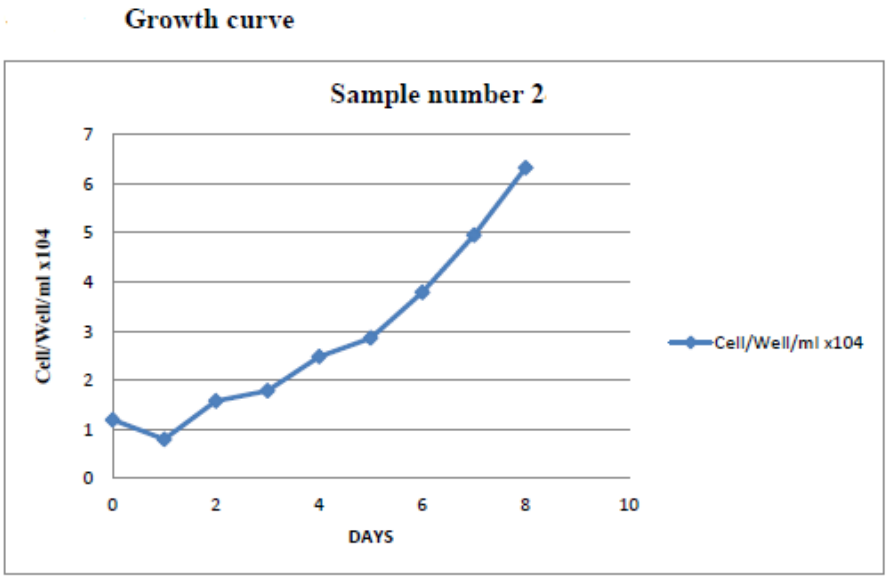
Table 13: EXPRESSION OF B7-H3 IN GINGIVAL FIBROBLASTS BY FLOWCYTOMETRY

S. NO	EXPRESSION%	MEAN	SD	CI
1	99.06	99.03	2.326	96.62-101.50
2	99.03		3.033	95.84-102.21
3	99.00		3.856	94.95-103.04
4	99.06		3.999	94.78-103.17
5	99.02		2.586	96.34-101.77

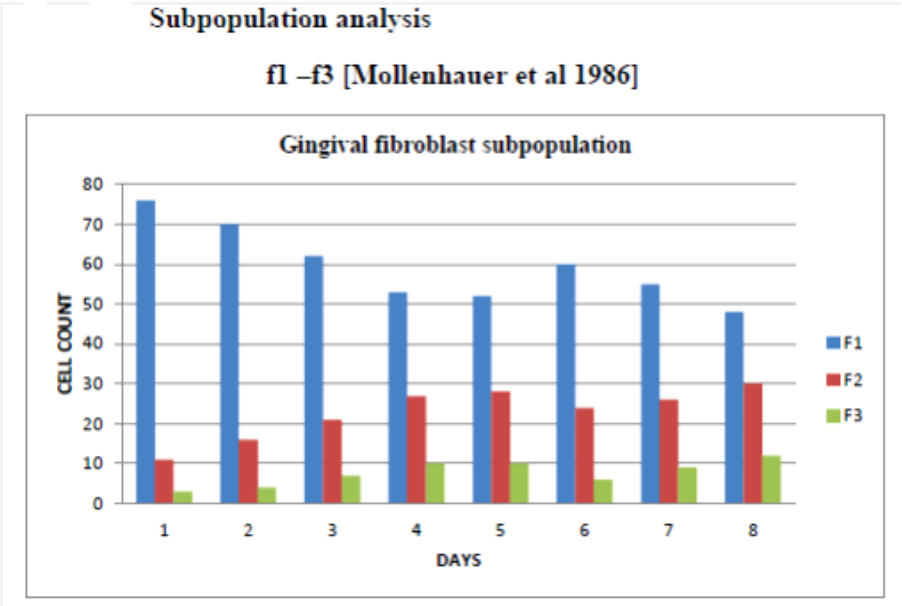
Table 14: EXPRESSION OF CD105 IN GINGIVAL FIBROBLASTS BY FLOWCYTOMETRY

S. NO	EXPRESSION %	MEAN	SD	CI
1	26.09	26.06	1.481	24.53-27.64
2	26.06		2.322	23.62-28.49
3	26.15		2.619	23.40-28.89
4	25.97		3.439	22.36-29.57
5	26.03		2.028	23.90-28.15

Graph 1:



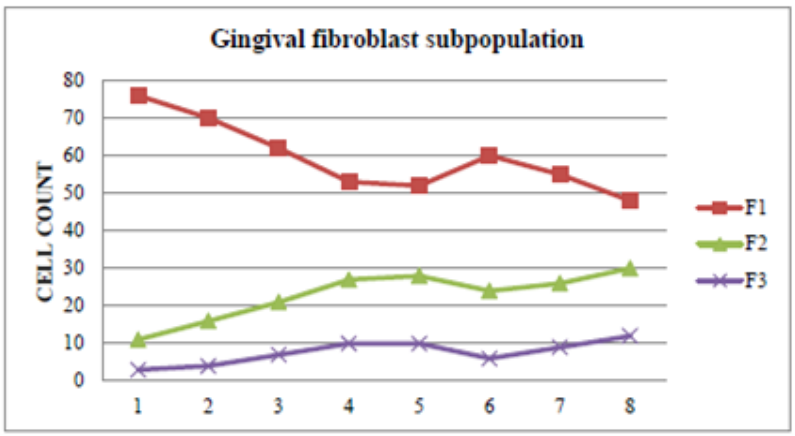
Graph 2:



Graph 3:

Subpopulation analysis

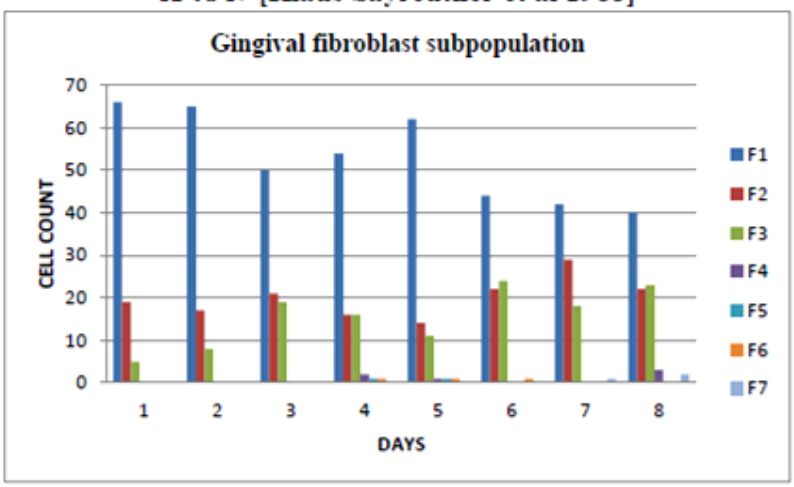
f1 –f3 [Mollenhauer et al 1986]



Graph 4:

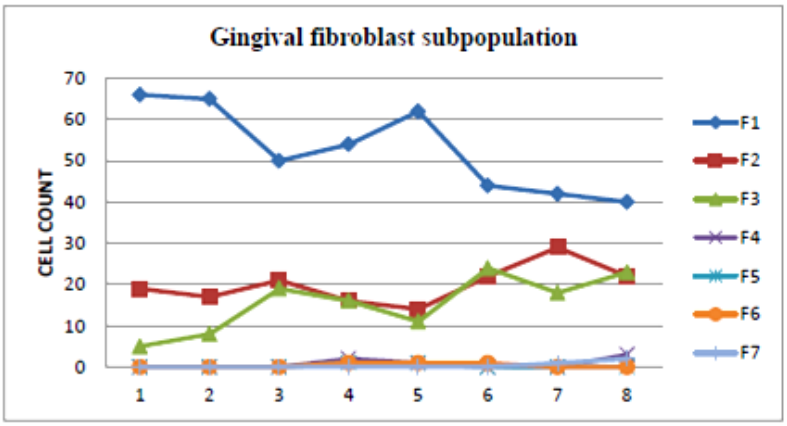
Subpopulation analysis

f1 to f7 [Klaus bayreuther et al 1988]



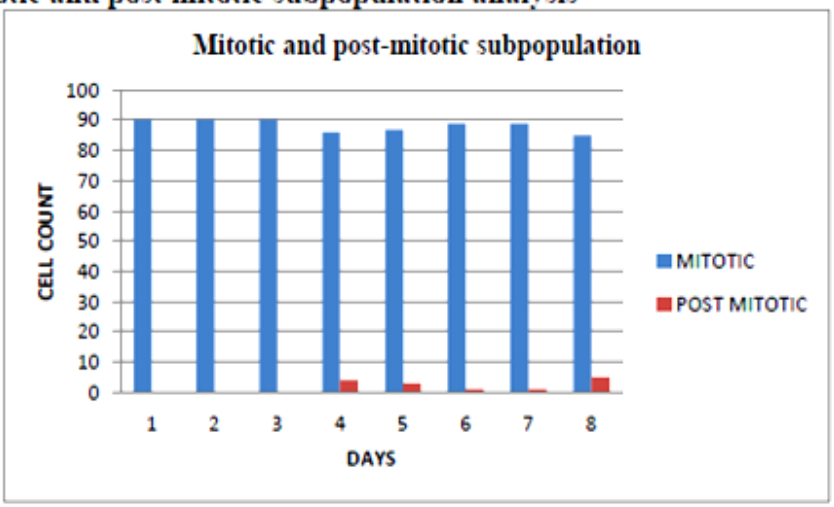
Graph 5:

Subpopulation analysis

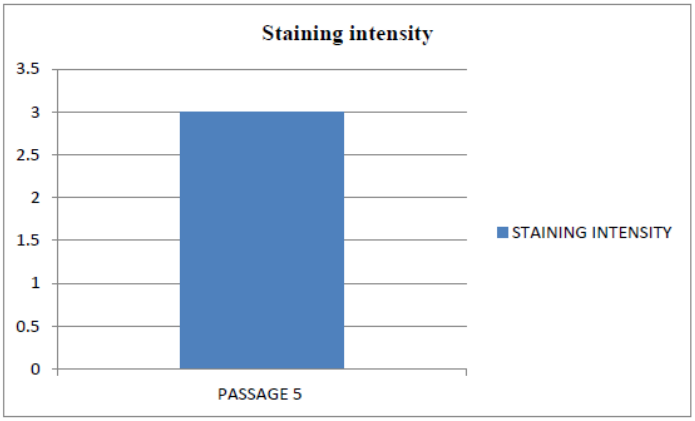


Graph 6:

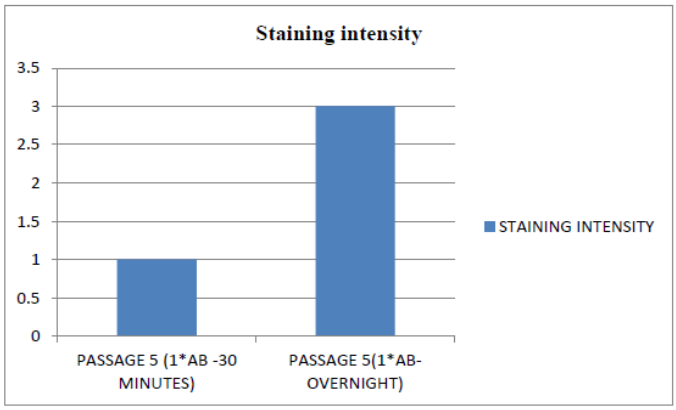
Mitotic and post mitotic subpopulation analysis



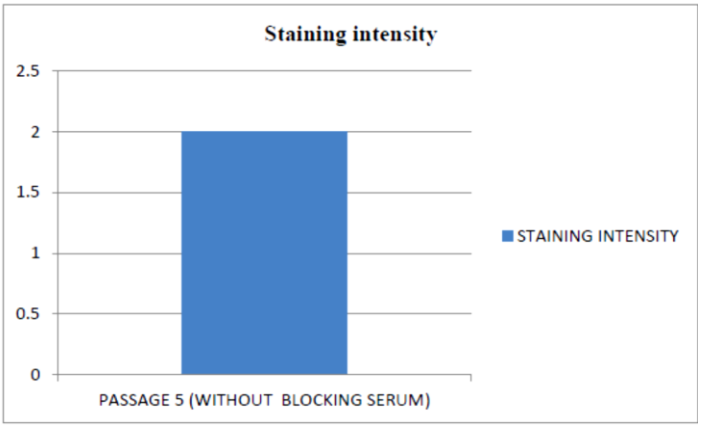
Graph 7: Immunocytochemical analysis of Vimentin in the 5th passage of culture



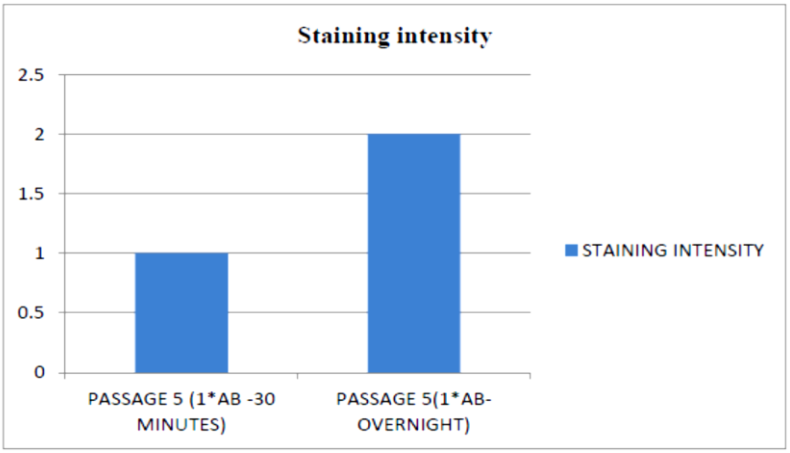
Graph 8: Immunocytochemical analysis of CD40 in the 5th passage of culture



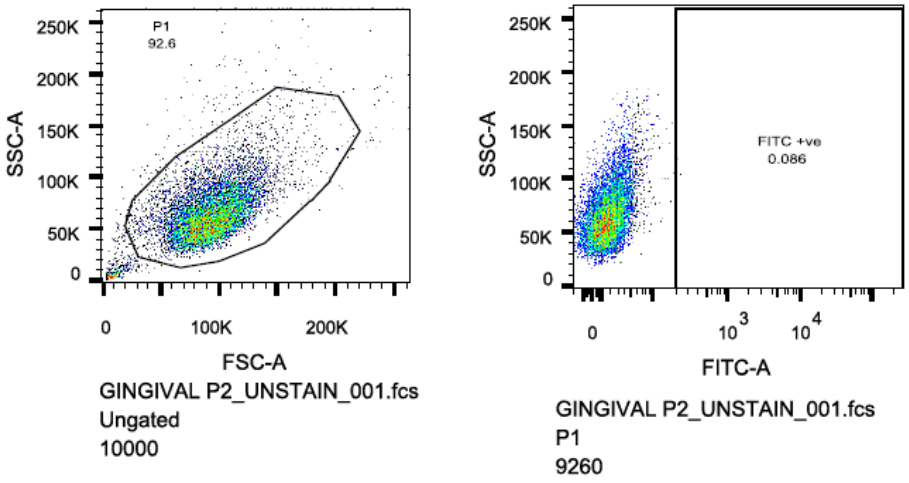
Graph 9: Immunocytochemcal analysis of B7-H3 in the 5th passage of culture



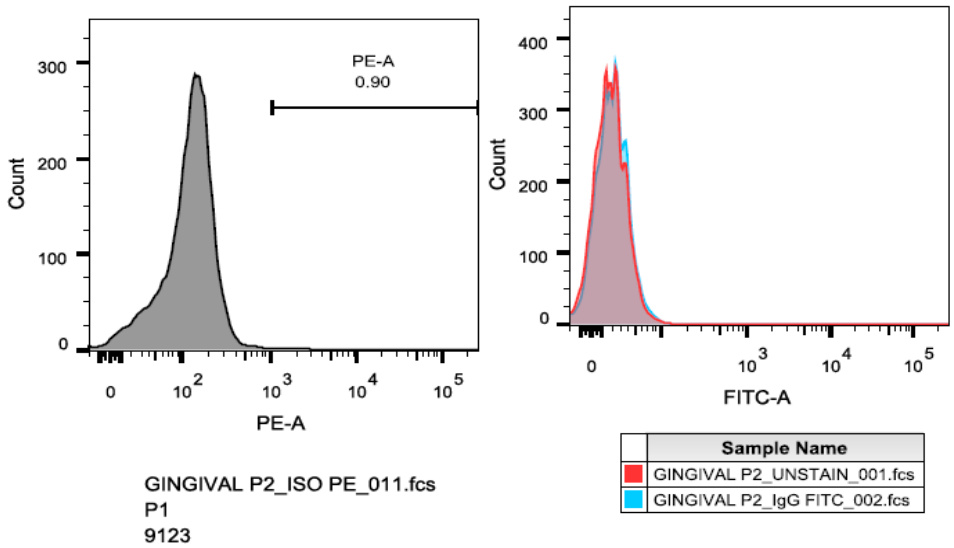
Graph 10:



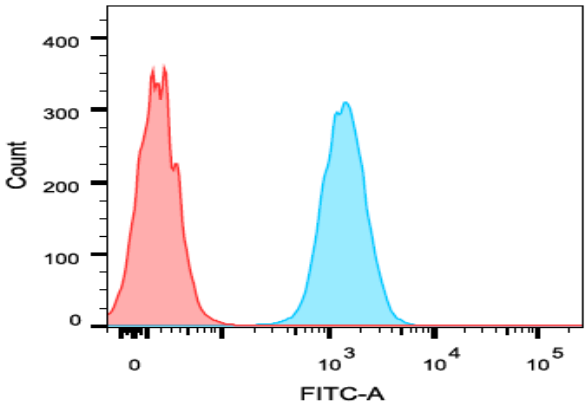
Graph 11: Unstained



Graph 12: Gingival fibroblasts – IgG-FITC

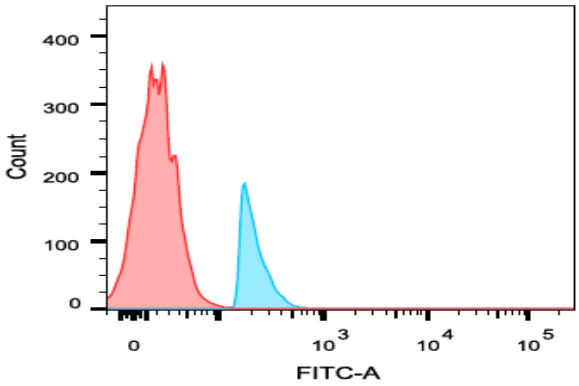


Graph 13: Gingival fibroblasts – CD 29-FITC



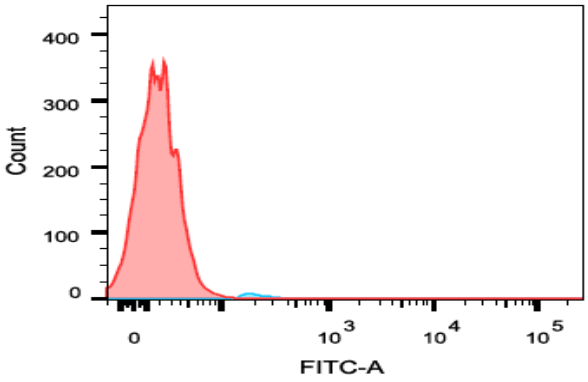
	Sample Name	Freq. of Parent
	GINGIVAL P2_UNSTAIN_001.fcs	92.6
	GINGIVAL P2_CD29 FITC_006.fcs	100.0

Graph 14: Gingival fibroblasts – CD105-FITC



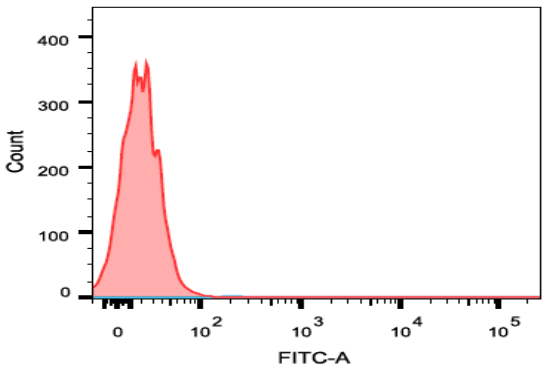
	Sample Name	Freq. of Parent
	GINGIVAL P2_UNSTAIN_001.fcs	92.6
	GINGIVAL P2_CD105 FITC_008.fcs	26.6

Graph 15: Gingival fibroblasts – CD14-FITC



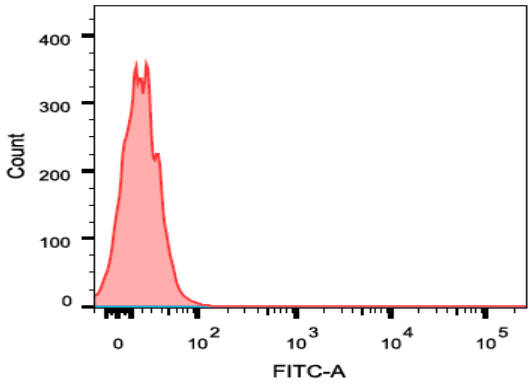
	Sample Name	Freq. of Parent
	GINGIVAL P2_UNSTAIN_001.fcs	92.6
	GINGIVAL P2_CD14 FITC_003.fcs	1.45

Graph 16: Gingival fibroblasts – CD34-FITC



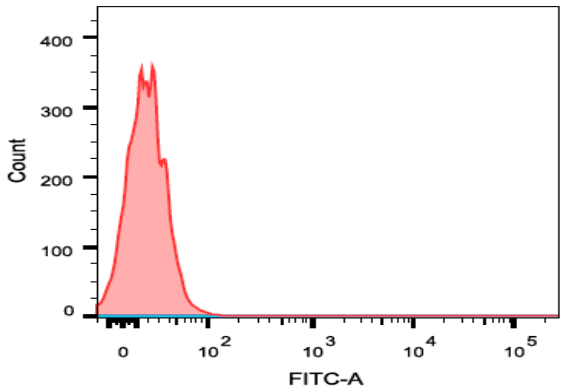
	Sample Name	Freq. of Parent
	GINGIVAL P2_UNSTAIN_001.fcs	92.6
	GINGIVAL P2_CD34 FITC_004.fcs	0.62

Graph 17: Gingival fibroblasts – CD45-FITC



	Sample Name	Freq. of Parent
	GINGIVAL P2_UNSTAIN_001.fcs	92.6
	GINGIVAL P2_CD45 FITC_005.fcs	0.36

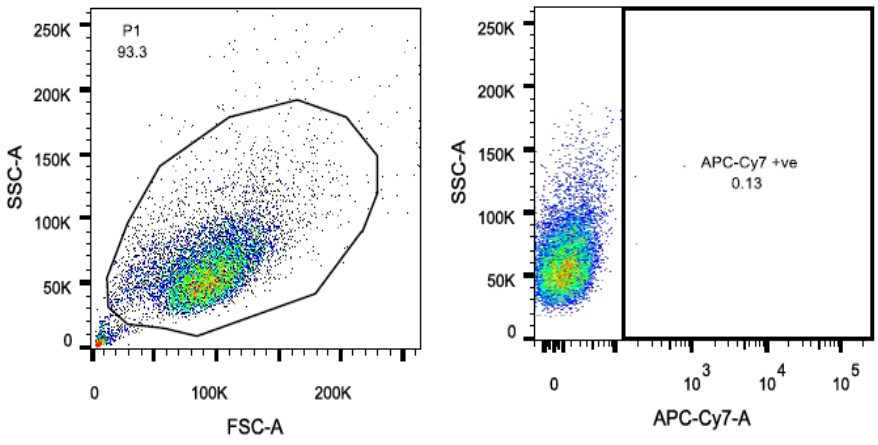
Graph 18: Gingival fibroblasts – HLA-DR-FITC



	Sample Name	Freq. of Parent
	GINGIVAL P2_UNSTAIN_001.fcs	92.6
	GINGIVAL P2_HLA-DR FITC_009.fcs	0.11

Graph 19: Gingival fibroblast – B7-H3 (CD 276) – APC-Cy7

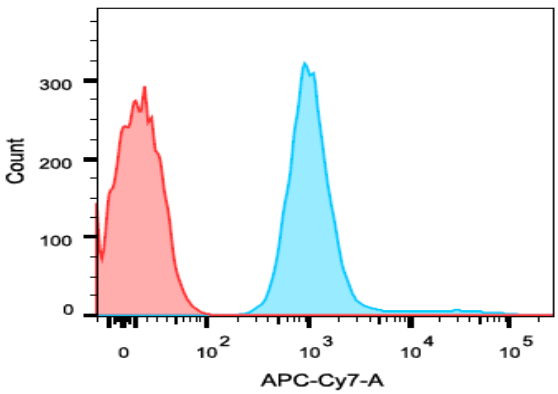
A) Unstained



GINGIVAL_UNSTAIN_001.fcs
Ungated
10000

GINGIVAL_UNSTAIN_001.fcs
P1
9328

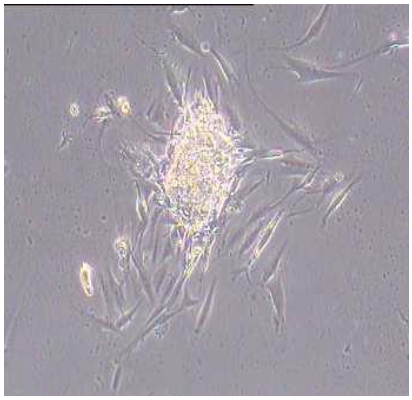
B) Stained – B7-H3 (CD 276) – APC-Cy7



	Sample Name	Freq. of Parent
	GINGIVAL_UNSTAIN_001.fcs	93.3
	GINGIVAL_CD276 APC CY7_002.fcs	99.3

Fig 1: Spindle shaped gingival fibroblasts growing out from collagenase disaggregated connective tissue.

(10X magnification)



(20X magnification)

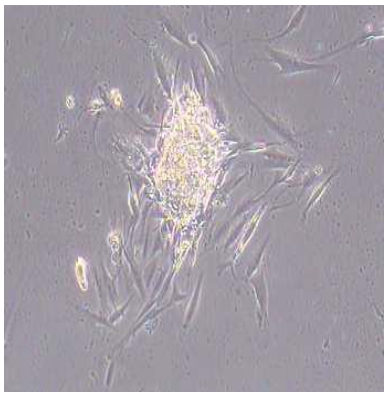


Fig 2: Sedimented Cell pellets after centrifugation (Red arrow)

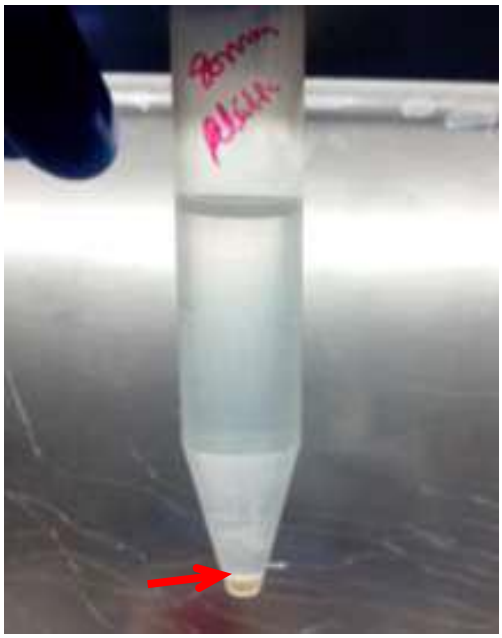


Fig 3: Fibroblasts growth -7th day of culture (20X magnification)

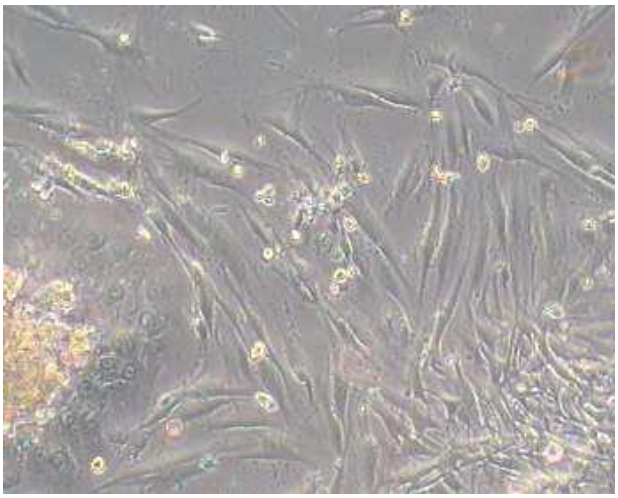
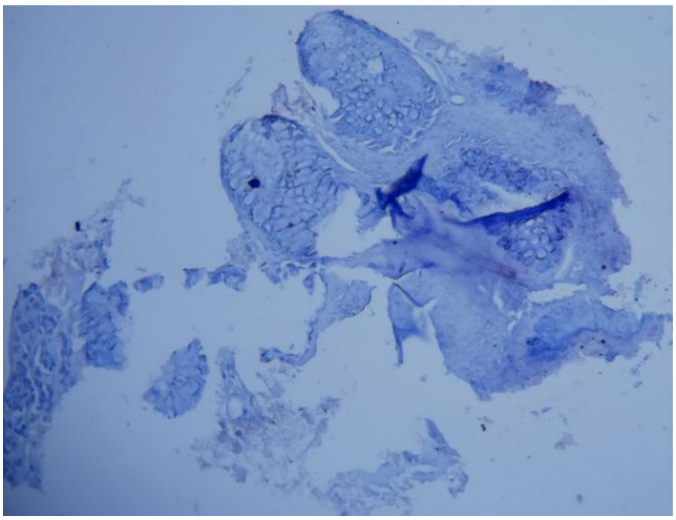


Fig 4: Positive control (Immunohistochemistry of prostate tissue sections)

A) On incubation without antibody



B) On incubation with antibody

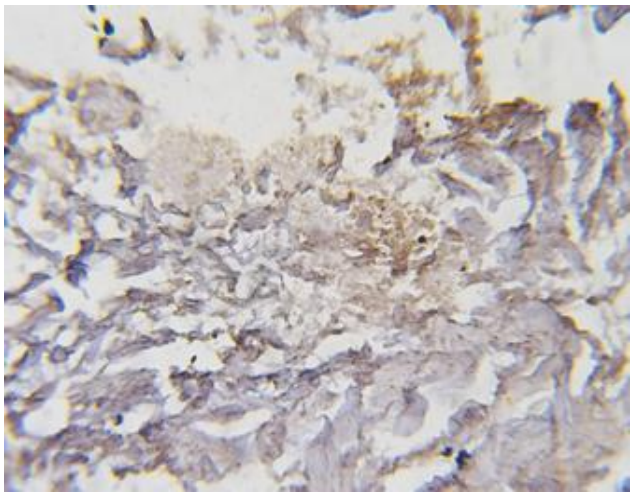


Fig 5: Unstained gingival fibroblasts (incubated without antibody)

(10X magnification)

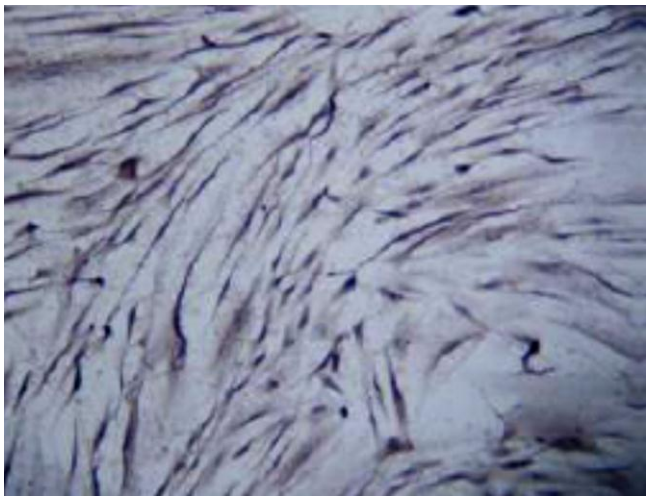
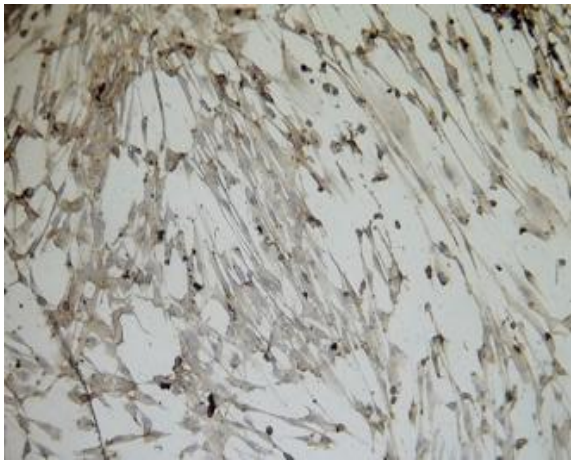


Fig 6: Gingival fibroblasts incubated with primary antibody

A) Overnight (10x magnification)



B) 30 minutes (10x magnification)

C) Overnight (40x magnification)

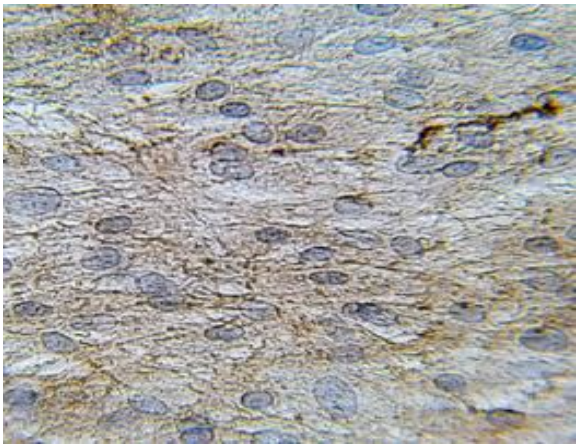
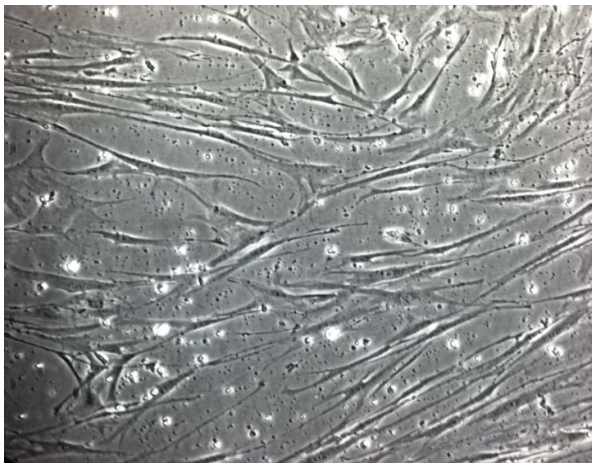


Fig 7: Gingival fibroblasts at p3 (20x magnification)



Discussion

DISCUSSION

The inflammatory response that occurs with the periodontal tissues includes both inflammatory/ immune cells and the stromal cells.[31]

Fibroblasts comprise the major anabolic cell population present in the periodontium. These resident cells present as a heterogeneous population of stromal cells, characterized by their spindle shaped morphology, flat oval nuclei and the lack of epithelial, vascular and leukocyte lineage markers.

The heterogenous fibroblast populations in the periodontium can be either of periodontal ligament or gingival origin. About 163 genes were found differentially expressed between periodontal ligament fibroblasts (PDLF) and gingival fibroblasts (GF).[120] GF have been reported to be more responsive to inflammatory stimuli when compared to PDLF.[5]

Gingival fibroblasts preserve the architecture of the connective tissue underlying the gingival epithelium and attaching to the alveolar bone. They are responsible for intracellular assembly of various extra cellular fibrillary and non fibrillary products such as, procollagen, pro-elastin and glycosaminoglycans.[176]

Dysregulation of fibroblast function may be observed both in tissue breakdown disorders such as periodontitis and in disorders characterized by increased turnover such as DIGO. (Drug Induced Gingival Overgrowth) [37, 145, 180]In the recent years they have also been known to maintain the homeostasis

of the adjacent cells and orchestrate the maintenance of inflammatory infiltrates. Fibroblasts are also shown to modify the quantity, quality and duration of the inflammatory infiltrate and play a critical role in the switch from an acute resolving condition to a chronic persistent inflammatory state.[129, 126]

It has been shown that fibroblasts secrete distinct patterns of matrix proteins, cytokines and chemokines. Apart from that, they are also known to express variable levels of co stimulatory molecules such as CD40 and vascular cell adhesion molecule (VCAM-1) suggesting a fundamental role in immune responses and disease processes.[23, 22]

The B7 family consists of immunomodulatory proteins that are required for the fine tuning of immune responses in addition to the primary signal provided by peptide-MHC complex. They belong to the immunoglobulin (Ig) superfamily of type I transmembrane proteins. Many studies have reported that the co stimulatory interactions that occurred between the B7 family ligands expressed on antigen-presenting cells (APC) and their receptors on T cells were essential for the growth, differentiation, and death of T cells. The molecules that have been included as members of the B7 family include – B7-1, B7-2, ICOS-L, B7-H1 (PD-L1), B7-DC (PD-L2), B7-H3 and B7-H4. The aim of this study was to evaluate if the co stimulatory molecule B7-H3 was expressed in gingival fibroblasts and thereby contribute to immune regulation. A previous study in this department has established the

presence of B7-H3 in gingival connective tissue but it was unclear from that study whether the expression of this protein was from immune cells or resident fibroblasts. Hence, this study was undertaken with a view to establish primary gingival fibroblast cultures and evaluate if B7-H3 was expressed on the cells.

Gingival fibroblasts were cultured from tissues that were obtained during gingivectomy that was done for the purpose of crown lengthening. Care was taken to ensure the absence of any clinical signs of inflammation in any of the tissues obtained.

Two samples could not be cultured perhaps as a result of contamination and standardization issues in our culture protocol. Gingival fibroblasts were obtained from all the other samples examined in the study. Their culture characteristics have been detailed in the results and are in accordance with previous studies.[150, 102] These authors have reported that GFs were not a fully homogenous group but consist of cells that vary in their mitotic activity and differential potential.

All the fibroblasts stained positively for Vimentin and CD40, through which they were confirmed to be gingival fibroblasts. Vimentin is a Type III intermediate filament of the fibroblasts which is an integral portion of the cell cytoskeleton. Hence it has been widely used as a marker for all fibroblast populations.[7] CD40 has been reported to be specific for gingival fibroblasts and has been use to distinguish them from other oral fibroblast populations like buccal, alveolar mucosal and periodontal ligament fibroblasts.[2]

The results of our immunocytochemical study showed that B7-H3 was expressed by all gingival fibroblasts. To the best of our knowledge, there are no previous reports available in literature to which we can compare our results with. Other studies however have reported the presence of other members of the B7 family such as PD-1 in T cells in periodontitis patients.[50] Similar results have also been reported in stromal cells in other peripheral tissues. [186]

Although immunocytochemistry established the presence of B7-H3 in gingival fibroblasts, the quantity and percentage of gingival fibroblasts that expressed this protein remained unknown. For this reason, the flowcytometry analysis was undertaken. Flowcytometry allows for simultaneous assessment of multiple markers. It was therefore possible to evaluate if gingival fibroblasts expressed other immune markers usually associated with leukocytes.

CD40 was not used as a marker for flowcytometry analysis as a complete positive staining was already established with immunocytochemistry.

The results of flowcytometry confirmed that B7-H3 was indeed expressed on 99.3% gingival fibroblasts that were examined. **(Table 13)**

To further characterize the accessory immune function, the gingival fibroblasts were evaluated for other markers related to differentiation and immune functions.

CD29 is known as β1 integrin (fibronectin receptor) which binds to the actin cytoskeleton.[125] The cells examined in the study showed a 100% positivity for CD29, thereby establishing that the gingival fibroblasts in our study were fully differentiated with complete cytoskeletal assembly.

CD105- Endoglin, is a Type I membrane glycopreotein located on cell surfaces and as a part of the Transforming Growth Factor β (TGF-β) receptor complex. It is known to play a crucial role in angiogenesis and is expressed on rapidly proliferating type of endothelial cells, lymphoblasts, fibroblasts and smooth muscle cells.[125] In this study, partial expression was detected on gingival fibroblasts (26.06%)

CD34 is known as the Hematopoietic progenitor cell antigen and is encoded by the CD-34 gene. It is a cell surface glycoprotein and functions in cell adhesion.[83]The gingival fibroblast cells did not express CD34 (0.62%) reflecting that it was not a progenitor cell, but more differentiated in nature.

CD14 is important for lipopolysaccharaide mediated immune response acting as a co receptor along with TLR-4. It is found to be expressed by macrophages, neutrophils and dendritic cells. [153] The cells in this study did not express CD14 (1.45%). This establishes that the function of gingival fibroblasts is primary anabolic with only a secondary immune function.

CD45 is also known as Protein tyrosine phosphatase receptor type C). It is characteristically expressed by T cells. It is specifically expressed in hematopoietic cells. [168] The cells in this study did not express CD45. (0.36%)

HLA-DR is expressed by APCs and is an MHC Class II cell surface receptor. The main function of this molecule is antigen presentation.[71] The cells did not express HLA-DR (0.11%)

To summarize these results, the flowcytometric analysis showed that all the gingival fibroblasts examined, expressed CD29 (100%), while there was a partial expression of CD105 (26.06%) **(Table 14)** and no expression of CD34. These results suggest that the gingival fibroblasts examined were differentiated, adult cells, with a full cytoskeletal assembly. Gingival fibroblasts did not express CD14, CD45 or HLA-DR, all of which are important for the immune responses generated by the primary immune cells such as dendritic cells and leucocytes. Gingival fibroblasts expressed B7-H3, but not other molecules present in the inflammatory/immune cells suggesting they have no role in antigen presentation, or in mediating LPS mediated immune pathways. Therefore, gingival fibroblasts may contribute to immune regulation only as secondary immune cells.

The exact role of these molecules in periodontal disease is difficult to ascertain with these results. We hypothesize that in health, gingival fibroblasts through B7-H3 expression, may provide signals for an increased basal inflammatory tone in response to commensal bacterial antigens. Earlier studies have revealed an increased expression of other members of the B7 family of molecules like B7-1 and B7-2 in healthy periodontal specimens.[59, 111]

Its role in periodontal disease at this stage remains speculative. However, B7-H3 has been found to play a pro-inflammatory role in other chronic inflammatory diseases.

Tran et al (2008) reported maximum B7-H3 expression in the lining layer of RA synovium in rheumatoid arthritis patients.[165] **Suh et al (2003)** revealed increasing severity of airway inflammation and increased T cell infiltration in murine studies using B7-H3 knockout mice.[154] **Kim J et al (2005)** reported B7-H3 expression in nasal epithelial cells of patients diagnosed with asthma and chronic rhino sinusitis.[87] **Zang et al (2007)** reported that increasing expression of B7-H3 in prostate cancer specimens correlates with an increased severity of disease and probability of recurrence.[182]

At present, factors determining periodontal disease progression are not well characterized. However, the presence or absence of tolerance has been implicated in disease progression. In normal circumstances, while the mRNA for the B7-H3 molecule is broadly distributed, tight control at the post-transcriptional level is imposed. Under a pathogenic environment, such as inflammation, the control is often aberrant.[90] It is thus possible that gingival fibroblasts may contribute to periodontal disease progression due to aberrant B7-H3 expression which may lead to increased T cell activation within the periodontal tissues.

Therapeutic implications of these results could be as follows: Targeting T cell activation has been a host modulation strategy that has been

adopted. These strategies might have to consider the role of the gingival fibroblasts in activating T cells through B7-H3.

Limitations of this study include

1. Antibodies only against B7-H3 molecule were used. As a result, the various other members of the B7 family were not evaluated.
2. Only healthy gingival fibroblasts were evaluated in this study. Therefore, its role in disease is difficult to establish. It is difficult to establish gingival fibroblast cultures from periodontitis sites because of the edematous and proteolytic environment in the gingival specimens. It is however possible to treat the gingival fibroblasts with LPS or with other cytokines to mimic the disease environment or co culture them with other immune cells.

Further studies conducted in this manner may help elucidate the exact role played by B7-H3 in the pathogenesis of periodontal disease.

Summary and Conclusion

SUMMARY AND CONCLUSION

This study was carried out to evaluate the expression of B7-H3 in healthy gingival tissue samples. The study population comprised of patients selected randomly from the Department of Periodontics, Ragas Dental College and Hospital, Chennai. A total of 7 patients were selected for the study. The 7 patients selected were periodontally healthy ranging from 20-50 years of age. Gingival tissues were obtained from periodontally healthy individuals who underwent a crown lengthening procedure. The collected tissue specimens were transported to the lab, the tissue was washed and a primary culture set up was established. The gingival fibroblasts were then evaluated for B7-H3 by using immunocytochemistry and these results were confirmed by flowcytometry.

The cells that were cultured in our study were characterized to be adult differentiated cells that expressed biomarkers that were characteristic of gingival fibroblasts. The gingival fibroblasts were found to express B7-H3 with moderate to intense (mean LI - 47.46) staining using immunocytochemistry. The expression of B7-H3 was confirmed with flowcytometry with 99.3% of the gingival fibroblasts examined expressing positivity.

These results suggest that gingival fibroblasts, through expression of B7-H3 may play an immunomodulatory role as well. However, the exact nature of the role played by B7-H3 in periodontal disease requires further elucidation.

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Annexures

ANNEXURE I

CONSENT FORM

I ……S/o, d/o, w/o……..aged about …….years…….residing at… …… do solemnly

And state as follows.

I have been explained about the nature and purpose of the study in which I have been asked to participate.

I give my consent after knowing full consequence of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I have been given the opportunity to ask questions about the procedure.

I also authorize the Doctor to proceed with the study and I will cooperate with the doctor.

I have also agreed to come for regular follow up for a period of at least one year.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

The doctor has explained the procedure to me and I have understood the same and signed my consent in(English/Tamil/Hindi/Telugu.....).

SIGNATURE OF THE PG STUDENT SIGNATURE OF THE PATIENT

SIGNATURE OF THE GUIDE: SIGNATURE OF THE HOD

ANNEXURE II

IMMUNOCYTOCHEMISTRY FOR CD40 AND VIMENTIN PROCEDURE FLOW CHART

Growing cells on APES coated slide

↓

Wash in PBS twice (5 minutes)

↓

Fix cells with methanol/acetone for 10 minutes

↓

Rinse with PBS thrice (5 minutes)

↓

Blocking with 0.1-1% endogenous peroxidase for 10 minutes

↓

Wash with PBS thrice (5 minutes each)

↓

Incubate with 1.5% blocking serum

↓

Blot excess serum

↓

Primary antibody added and incubated for 30 minutes or overnight

↓

Wash in PBS thrice (5 minutes each)

↓

Secondary antibody added and incubated in an enclosed hydrated container (30 minutes)

↓

Wash in PBS thrice (5 minutes each)

↓

Incubate with AB enzyme reagent (30 minutes)

↓

Wash in PBS thrice (5 minutes each)

↓

Incubate with 1-3 drops of peroxidase substrate (10 minutes)

↓

Wash in de-ionised water (5 minutes)

↓

Stained with Gill's formulation #2 hematoxylin (5 - 10 seconds)

↓

Wash in several changes of deionised water

↓

Destain in acid alcohol

↓

Mount the slides using 1-2 drops of DPX

↓

Observe the slides under the LM and grade

ANNEXURE III

CASE HISTORY PROFORMA

RAGAS DENTAL COLLEGE AND HOSPITALS, CHENNAI.

DEPARTMENT OF PERIODONTICS

PROFORMA

NAME: AGE: SEX: DATE:

ADDRESS: OCCUPATION:

OP.NO:

CHIEF COMPLAINT:

PAST DENTAL HISTORY:

MEDICAL HISTORY:

PERSONAL HABITS:

INTRAORAL EXAMINATION

A. HARD TISSUE EXAMINATION:

B. SOFT TISSUE EXAMINATION:

- GINGIVAL FINDINGS

- DENUDED ROOTS (MILLER'S CLASSIFICATION)

PLAQUE SCORE:

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

BLEEDING SCORE:

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

Measurement of Probing Depth and Clinical Attachment Loss

																CAL
																PD
DB MB MB DP MP MP	DB MB MB DP MP MP	DB MB MB DP MP MP	DB MB MB DP MP MP	DB MB MB DP MP MP	DB MB MB DP MP MP	DB MB MB DP MP MP	DB MB MB DP MP MP	MB MB DB MP MP DP	MB MB DB MP MP DP	MB MB DB MP MP DP	MB MB DB MP MP DP	MB MB DB MP MP DP	MB MB DB MP MP DP	MB MB DB MP MP DP	MB MB DB MP MP DP	
																PD
																CAL
18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28	
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38	
																CAL
																PD
DL ML ML DB MB MB	DL ML ML DB MB MB	DL ML ML DB MB MB	DL ML ML DB MB MB	DL ML ML DB MB MB	DL ML ML DB MB MB	DL ML ML DB MB MB	DL ML ML DB MB MB	ML ML DL MB MB DB	ML ML DL MB MB DB	ML ML DL MB MB DB	ML ML DL MB MB DB	ML ML DL MB MB DB	ML ML DL MB MB DB	ML ML DL MB MB DB	ML ML DL MB MB DB	
																PD
																CAL

ANNEXURES IV

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA.
Tele : (044) 24530002, 24530003 - 06. Principal (Dir) 24530001 Fax : (044) 24530009

TO WHOM SO EVER IT MAY CONCERN

Date: 04-01-2016
Place: Chennai

From
The Institutional Review Board,
Ragas Dental College & Hospital,
Uthandi,
Chennai – 600119.

The thesis topic **'EVALUATION OF EXPRESSION OF B7-H3 IN GINGIVAL FIBROBLASTS – A CELL CULTURE, IMMUNOCYTOCHEMISTRY AND FLOWCYTOMETRY STUDY'**, submitted by **Dr. NIKHITA REBEKAH DAVID** has been approved by the institutional review board of Ragas Dental College & Hospital on 5th May, 2014.

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