Evaluation of mRNA expression of the Transcription factors of Th1 and Th2 subsets (T- bet and GATA-3) In Periodontal Health and Disease

Dissertation submitted to

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PERIODONTICS

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CERTIFICATE

This is to certify that this dissertation titled **"Evaluation of mRNA expression of the Transcription factors of Th1 and Th2 subsets (T- bet and GATA-3), In Periodontal Health and Disease"** is a bonafide record of work done by **Dr. N.Rajesh** under my guidance during the study period of 2009-2012.

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

APC - Antigen Presenting Cell

CAL - Clinical Attachment Level

CD - Cluster of Differentiation

CTL - Cytotoxic T Lymphocytes

CTLA - Cytotoxic T-Lymphocyte Antigen

CMI - Cell Mediated Immunity

DC - Dendritic Cell

DTH - Delayed Type Hypersensitivity

foxp3 - Forkhead box protein p3 transcription Factor

GMC - Gingival Mononuclear Cells

GCF - Gingival Crevicular Fluid

GATA-3 - Trans-acting T-cell-specific transcription factor *GATA-3*

HLA - Human Leukocyte Antigen

HLX - H2.0-like homeobox 1

ICAM - Intercellular Adhesion Molecule

Ig - Immunoglobulin

IL - Interleukin

IFN - Interferon

LFA - Lymphocyte Function-associated Antigen

LPS - Lipopolysaccharide

MHC - Major Histocompatibility Complex

MMP - Matrix Metalloproteinase

NK - Natural Killer

Pg - Porphyromonas gingivalis

PGE2 - Prostaglandin

PBMC - Peripheral Blood Mononuclear Cells

PBS - Phosphate Buffered Saline

PMN - Polymorphonuclear neutrophils

PPD - Periodontal Probing Depth

PAMP - Pathogen-Associated Molecular Patterns

PRR - Pathogen Recognition Receptor

RA - Rheumatoid Arthritis

RANKL - Receptor Activator of Nuclear Factor- κB ligand

STAT - Signal Transduction and Activator of Transcription

TCR - T cell receptor

TGF - Transforming Growth Factor

Th - Helper T cell

TNF - Tumor Necrosis Factor

Treg - Regulatory T cell

Tc - Cytotoxic T cell

Th - Helper T cell

To - Naive T cell

T bet - T box expressed activated T cell

TIMMP - Tissue Inhibitor of Matrix Metalloproteinase

TLR - Toll like receptor

SD - Standard deviation

Abstract

Background: T cells play a prominent role in the etiopathogenesis of periodontal disease. The Th1/Th2 paradigm which has been used to explain the pathogenic mechanisms involved in periodontal disease, was based on the cytokine profile. Establishment of Th1 and Th2 subsets largely depends on its transcriptional regulation. Hence the aim of the study is to evaluate the transcriptional regulation of Th1 and Th2 subsets (T- bet and GATA-3), in Periodontal Health and Disease.

Materials and methods: Ten gingival tissue biopsy samples from each group of patients (periodontal health-Group A and periodontal disease-Group B) were obtained. The samples were processed for real time reverse transcription polymerase chain reaction to detect the mRNA expression of T-bet and GATA-3 in periodontal health and disease samples.

Results: The mRNA expression of the master transcription factor of Th1 subset, T- bet showed a marginal increase of about 1.31 fold, which was insignificant, while the GATA-3 levels showed a 4.39 fold decrease from disease to health.

Conclusion: The results of this study indicates that the advanced periodontal lesions are more likely to be dominated by Th1, rather than Th2.

Key words: T-bet, GATA-3, real time RT - PCR

Introduction:

Periodontitis is an inflammatory disease of the supporting tissues of the teeth caused by specific micro organisms or groups of specific micro organisms resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both.[105]It is now recognised that although periodontitis is microbial in origin, destructive processes are mediated through an exaggerated host response resulting in increased proinflammatory cytokine release. The imbalance between the invading micro organisms and the host response results in periodontal disease. [127]

The central role played by T cells in the etiopathogenesis of periodontal disease was recognized following the seminal work of Ivanher and Leanyi.[62] However, with a greater understanding of their biological functions, T cells are now believed to be involved in the homeostasis of periodontal tissues,[41] modulation of the inflammatory/immune responses[12] and mediation of the bone loss observed in periodontal disease.[150]

In periodontal disease, cytokines are central to the pathogenic process involved in disease activity. It has been postulated that "appropriate" cytokine production results in protective immunity, while "inappropriate" cytokine production leads to tissue destruction and disease progression.[42]

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.

Originally, the presence of two different subsets of Th cells-Th1 and Th2 was described by Parish and Liew. Later, Mossman and Coffman [106] delineated two distinct cytokine profiles associated with the Th1 and Th2 cells.

Macrophage activity leading to osteoclastogenesis has been reported to be associated with the bone destruction observed in periodontal disease.[146,149]
Th1 cells are believed to have evolved to afford protection against the intracellular pathogens. These cells produce the interferons (IFN) that are involved in macrophage activation, which in turn plays an important role in phagocytosis, complement fixation and opsonization.[114] Th2 cells are thought to have evolved as protection against parasitic helminths. These cells produce IL-4 in addition to IL-5 and IL-13; cytokines that are involved in immunoglobulin (Ig) class switching in B cells. The importance of IgG that is produced as a result of this class switching in periodontal disease pathogenesis has been well-documented.

Naive T-Helper cells can be differentiated into either TH-1 cells or TH-2 cells, depending on their cytokine nature and Transcriptional factors.[92,94]

Glimcher in 2007,[49] stated that the Th1 cell expresses the IL-12 receptor subunit IL-12Rβ2, which further commits a cell to proceed along this differentiation program. This process is dependent on the transcription factor STAT-4, which is activated by IL-12, and also on T-bet, which is considered the master regulator of the Th1 lineage.[49]

Conversely, when a newly activated Th cell is exposed to IL-4, differentiation to a Th2 phenotype occurs. Th2 cells secrete IL-4, IL-5, and IL-13, which are co-regulated transcriptionally.

Yamazaki.K *et al*, in 2007, stated that Th2 development is dependent on the transcription factors STAT-6, GATA-3, and c - maf. Th1 and Th2 development is mutually antagonistic and self-reinforcing, in part because IL-4 and IFN-γ antagonize one another at cellular and molecular levels.[162]

Although a number of studies have documented the preponderance of either the Th1/Th2 signature cytokines in tissues, saliva, GCF, however there are fewer reports on the transcriptional activity within the gingival tissues.

Aims & Objectives:

The aim of this study is to evaluate the mRNA expression of the transcription factors of Th1 and Th2 subsets namely T-bet and GATA-3 respectively, from the gingival tissue samples of periodontally healthy and diseased patients.

REVIEW OF LITERATURE

Periodontitis is defined as an inflammatory disease of the supporting tissues of the teeth caused by specific micro organisms or groups of specific micro organisms resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both (**Armitage GC et al**.)[9, 11]

Chronic inflammatory periodontal disease results from the inflammatory response to bacteria in dental plaque and may either remain confined to gingival tissues or progress leading to attachment loss. Disease progression is due to combination of factors including the presence of periodontopathic bacteria and high level of pro-inflammatory cytokines, MMPS, PGE2 and low levels of anti-inflammatory cytokines (IL-10, TGF-β, and TIMMPS).

The adaptive immune response is under the control of T cells which regulate B cell/ plasma cell differentiation and antibody production. Clearance of bacteria by neutrophils may depend upon the presence of IFN-γ and further enhanced by protective antibodies which in turn control by the types of cytokines produced by T cells.(**Page R.C , Offenbacher S , Schroeder H.E, Seymour G.J**)[105]

Cytokines are characterized by their pleiotropism and pluripotentiality. Certain cytokines-including interleukin-1 (IL-1), IL-6, IL-8, IL-12, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ)-serve predominantly pro-inflammatory functions (**Dinarello, 1987**).[28] These cytokines are produced by or act on mononuclear phagocytes and other cell types to upregulate adhesion molecules, immunoglobulin Fc receptors, nitric oxide synthesis, eicosanoid and metalloprotease production, and cytokine secretion. They also promote connective tissue and endothelial cell activation. Other cytokines generally function in an anti-inflammatory role. These include IL-1 receptor antagonist (IL-lra), IL-13, and transforming growth factor-β(TGF-β,) (**Zurawski, 1994**).[179]

Reinherz, 1985,[110] documented that T cells are involved in nearly all immunoregulatory interactions both in vivo and in vitro. Prothymocytes migrate to the thymus gland where they are processed, become functionally competent, and then exported into the peripheral lymphoid compartment. There are distinct subpopulations of peripheral T cells and the delicate balance between effector and regulatory subsets that normally exists is required for immune homeostasis.

Over two decades ago, **Mosmann and Coffman 1986,**[92] proposed a model wherein T helper (Th) cells are divided into functional subsets on the basis of cytokine secretion, termed Th1 and Th2. Th1 cells secrete IFNγ, and

thereby activate macrophages, NK cells, and CD8+ T-cells (collectively termed "cell-mediated immunity", or CMI). Th1 cytokines also influence B-cells to secrete opsonizing antibody isotypes that further enhance antigen uptake and presentation to T-cells. Until recently, Th1 cells were also thought to be the major effectors of immunity to extracellular pathogens and autoimmunity. Th2 cells, in contrast, mediate humoral immunity, including production of IgE and activate mast cells, which drive immune responses to helminths.

Numerous studies have attempted to delineate the Th1-Th2 cytokine profile in chronic periodontitis in humans. **Page and Schroeder, et al 1976**,[105] stated that during the early stage, the inflammatory infiltrate is mostly T-cells, whereas in the established lesions, B-cells become the most common inflammatory cells.

While some controversy still exists regarding these profiles, the fact is that, the stable periodontal lesion is identical to a delayed type hypersensitivity reaction. This suggests that stable lesions are mediated by Th1 cells and progressive lesions involving B cells and plasma cells are mediated by Th2 cells. In chronic periodontitis the T cell receptor (TCR) seems to direct the Th1 response while low affinity TCR involvement tends towards Th2 response. This TCR affinity in chronic periodontitis has not been

investigated so the mechanism which controls Th1, Th2 profiles includes the nature of antigen presentation and innate immune response.

Innate versus adaptive immunity:-

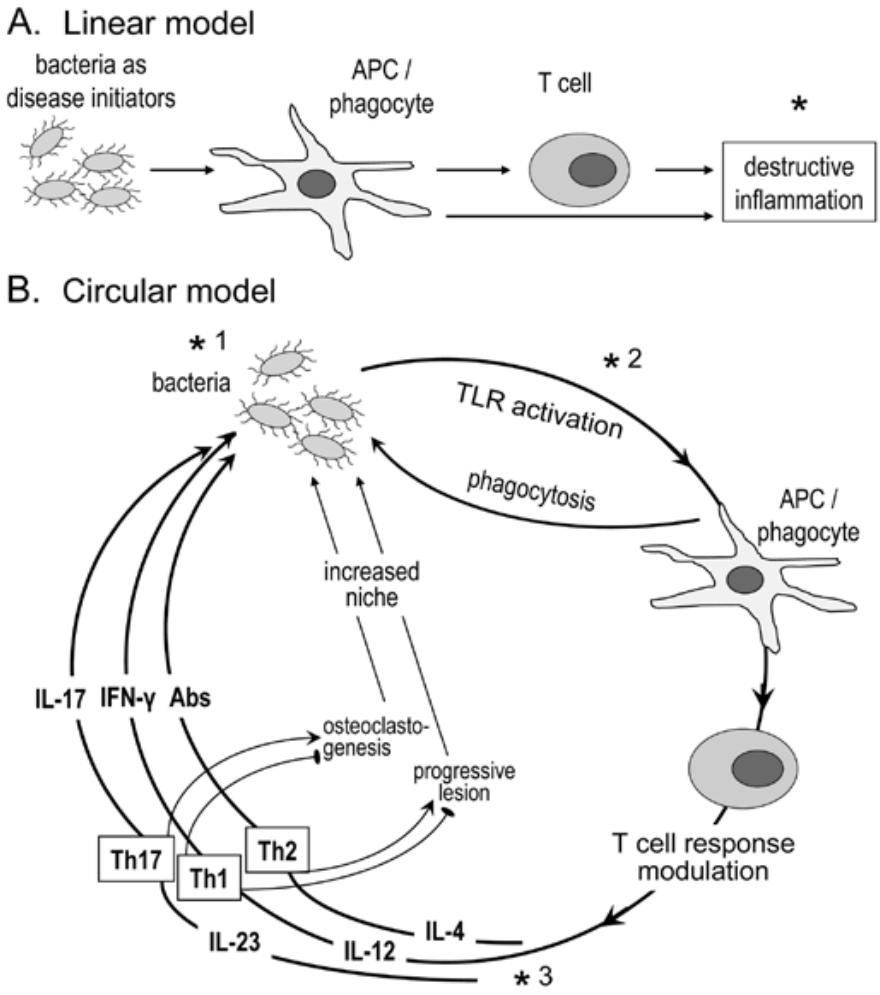
Since birth, our immune system is constantly encountered with self-antigens, innocuous antigens and foreign pathogens, such as airborne and food-borne viruses, bacteria and parasites. To stay healthy, complex immune strategies have evolved in mammals to maintain self-tolerance and to defend against foreign pathogens. To accomplish such a task, the immune system has developed mechanisms to efficiently cope with these insults in the living species.

The two fundamental arms of the immune response, innate and adaptive immunity, together form a defensive front against pathogens. Innate immunity evolved earlier and is mediated by cells such as macrophages and dendritic cells (DC) and is specific only to a few classes of microorganisms. The later evolved adaptive immunity mediated by T and B cells and enhances pathogen eradication in vertebrates by adding antigen specificity and memory onto pre-existent innate immunity.

Immunity- the state of protection from infectious disease has both nonspecific and specific components. The nonspecific component, innate immunity, provides the first line of defence during the critical period just after the host's exposure to a pathogen. Phagocytic cells such as macrophages play an important role in many aspects of innate immunity. In contrast, the specific component, adaptive immunity, displays a high degree of specificity as well as the remarkable property of "Memory".

Exposure to the same antigen sometime later in future results in a memory response of the immune system to the second exposure more quickly than the first. This response is stronger and often more effective in neutralising and clearing the pathogen. If an invading microorganism eludes innate mechanisms or is not cleared by them, the specific immune response of adaptive immunity is triggered. Adaptive and innate immunity do not operate independently of each other, they function as a highly interactive and cooperative system, producing a total response more effective than either could alone.

Concepts in periodontal disease pathogenesis:



Salvi and Lang, 2005,[117] documented that in a linear model (A), the bacteria are seen as initiators of the inflammatory process. Disease progression depends exclusively on the host response, which offers a target (*) for therapeutic intervention (*e.g.*, anti-inflammatory agents) .

The circular model (B) contends that bacteria are necessary for both initiation and progression of the disease, by constantly shaping the T-cell

response through differential TLR-mediated activation of APC and secreted cytokines.

The innate and adaptive host response, in turn, determines the fate of the infection, and consequently whether inflammation will progress to disease or be controlled. Although the role of the individual effector Th subsets is debatable, **Gemmell *et al.*, 2002,** [47] stated that Th2 cells have been associated with non protective antibody responses and progressive periodontal lesions, and Th1 with stable lesions.

Th1 cells are thought to protect through IL-12/IFNγ stimulated cell-mediated immunity (**Alayan *et al.*, 2007;**) [4] and by inhibition of osteoclastogenesis (**Gowen *et al.*, 1986; Horwood *et al.*, 2001**).[51,52] In contrast, **Sato *et al.*, 2006,**[121] documented that Th17 cells have been implicated as a specialized osteoclastogenic subset that links T-cell activation to bone loss. Periodontal bone loss and soft-tissue alterations offer new niches for colonization (generation of deep periodontal pockets) and thus facilitate bacterial overgrowth; this model offers three types of targets for therapeutic intervention: antimicrobial (*1), TLR-based modulation of APC responses (*2), and cytokine-based control of T-cell activation (*3).

PERIODONTAL DISEASE AND CELL-MEDIATED IMMUNITY:

Periodontitis is an infection-driven chronic inflammatory disease affecting the integrity of tooth-supporting tissues. Subgingival bacterial pathogens are essential for the initiation and progression of the disease, although it is the resulting host reaction that primarily mediates tissue damage (**Baker, 2000; Taubman *et al.*, 2005**).[13,146]

Gemmell *et al.*,2007; Kinane *et al.*, 2007,[41,44] had reviewed that the periodontal host response is highly complex; it contains both protective and destructive elements, and may be proactively modified by immune-subverting pathogens. Despite almost four decades of intensive research, the precise role of the host response in periodontitis and how it can be harnessed therapeutically are far from resolved.

In one of the first reports by **Ivanyi and Lehner, 1970** [62] addressing a role for CMI in periodontitis, the authors concluded that "the cell mediated immune response to some oral micro-organisms may play a protective or aggressive part in the pathogenesis of periodontal disease". In a case with inflammatory diseases, CMI would be expected to play a pathological role in periodontitis.

However, the experimental studies conducted by **Ivanyi and Lehner, 1970** [62] have shown the protective function of CMI because they observed

suppressed CMI in individuals with advanced periodontitis compared with those with mild disease. This statement is still relevant, despite a flurry of discoveries and advancements in their understanding of T-cell subsets and their cytokines, as well as the innate immune mechanisms involved in their regulation.

Early immunohistological studies by **Page and Schroeder, 1976; reviewed by Gemmell *et al.*, 2007**[105] in humans established that the early periodontal lesion (gingivitis) is characterized by increased numbers of T-cells and macrophages, whereas the progressive lesion (leading to periodontitis) is associated with increased infiltration by B-cells and plasma cells.

Evans *et al.*, 1989[31] had stated that after periodontal treatment there is suppressed potential for the induction of CMI in advanced periodontitis.

It was thus proposed that periodontal bacteria play an active role in suppressing CMI, thereby inducing the transition from an early/stable lesion to a progressive/advanced lesion.

T-CELL-MEDIATED IMMUNITY IN THE PERIODONTIUM:

It is generally accepted that systemic immune status can affect local immune responses, including the periodontium, and that periodontal disease progression is critically determined by the nature of inflammatory responses generated in response to the challenges from the subgingival micro-organisms (or biofilm).

The study of various genetic knockout and immunodeficient mouse strains and the use of advanced cellular and molecular biology techniques have brought the new understanding and insights into the nature of, and regulations associated with, inflammatory events in the periodontium. **Baker *et al.*, 1994, 1999, 2002,**[13,14] In addition to the protective and somewhat unclear inflammatory and anti-inflammatory activity of humoral Ig immunity in the periodontium, it is now clear that when the host's T-cell-mediated immune system is absent or deficient during the course of periodontal infection, compared with that of the immunocompetent host, there is much less tissue inflammation and alveolar bone destruction.

Seymour and Gemmell, 2001; Gemmell *et al.*, 2002,[127] documented that CD8+ T-cells may be involved in killing and removing some bacteria-infected or damaged host cells, they do not directly mediate the periodontal destruction *in situ*. Importantly, CD4+ T-cells and other Phagocytic lineage or innate immune cells are critically involved in effective defenses to eradicate the invading pathogens and directly participate in tissue

inflammatory responses and alveolar bone destruction, in particular, through the cytokines they produce.

Seymour and Gemmell, 2001; Taubman and Kawai, 2001,[126] stated that cytokines expressed locally in the periodontal tissues and by the inflammatory cells contribute to the state of protective *vs.* destructive phases of the disease progression.

Baker *et al.*, 1999; Garlet *et al.*, 2003 [13,39] in their experimental studies had clearly shown that, based on cytokine expression profiles, (i) both Th1 and Th2 cells and cytokines are often present simultaneously in the infected periodontal tissues , and (ii) pathogen-reactive Th1 cells and the cytokines they produce (*i.e.*,IFN-γ) can mediate the active inflammatory response associated with tissue and alveolar bone destruction (**Kawai *et al.*,2000; Taubman and Kawai, 2001**).[72]

Cytokines are of major importance in periodontal disease progression. It is generally agreed that control of the Th1/Th2 balance is central to the immunoregulation of periodontal disease. There is increasing evidence in humans that the stable periodontal lesion is mediated by Th1 cells, while the progressive lesion sees a shift toward Th2 cells. Equally, there is conflicting evidence, mainly in animal models, that bone loss is mediated by Th1 responses, and that Th2 responses are protective. In the presence of IL-12,

IL-18 induces Th1 responses while, in the absence of IL-12, it promotes Th2 responses. It is clear, therefore, that since IL-18 has the ability to induce either Th1 or Th2 differentiation, and it becomes important to consider its role in periodontal disease.

Key Cytokines in Periodontal Disease:

Although individual PRR (pattern recognition receptor) ligands in various periodontal pathogens have the potential to modulate the T-cell response, the pathogenesis of periodontitis might be too complex by distinct T-cell subsets. Since this disease is polymicrobial, pathogens may modulate the T-cell response to promote their own adaptive strength, and the net immune response is mediated by all the microbes represented in the biofilm. Therefore, it may not be possible to dissect dominant Th1, Th2, activity patterns reliably among collected samples of diseased periodontal tissue. Accordingly, it might be simpler and more productive to consider the roles of individual Th1, Th2, cytokines in periodontal infection.

IL-12:

Trinchieri *et al.*, 2003[153] documented that IL-12p70 plays a key role in mediating bacterial clearance through the induction of IFN-γ, which in turn activates the bactericidal function of macrophages. **Karp *et al.*, 1996,**[71] had shown that several pathogens have developed mechanisms for IL-12

suppression. Several animal studies have examined the role of IL-12 in protection against periodontal pathogens.

Alayan *et al.*, 2007,[4] had shown that IL-12p40 deficient mice exhibit a reduced inflammatory cell infiltrate, but increased tissue destruction, upon subcutaneous challenge with *P. gingivalis*. In contrast, mice deficient in Th2 cytokines, such as IL-4 or IL-10, did not display increased susceptibility to *P. gingivalis*-induced tissue destruction. Moreover, IL-12p40 deficient mice are more susceptible to naturally occurring murine periodontitis than are wild-type controls. These findings could be interpreted to mean that IL-12 is important for the immune and inflammatory responses that control *P. gingivalis* infection.

In another study, **Johnson and Serio, 2005,**[66] shown that IFN-γ and IL-12 were negatively correlated with gingival sulcular depth, in contrast to other cytokines (IL-6 and IL-18) that displayed positive correlations and were thus thought to contribute to non-resolving inflammation.

Hajishengallis *etal.*, 2007; Wang *et al.*, 2007,[54] was hypothesized in these studies that periodontal infection and resulting inflammation may persist due to decreased IL-12 levels. In addition, *P. gingivalis* possesses a mechanism for inhibiting the production of IL-12 and IFN-γ in humans and mice and for degrading human IL-12 (**Yun *et al.*, 2001**). [168]

Gemmell *et al.*, 2006,[43] documented *P. gingivalis* has the potential to induce a immunosuppression of CD4+ and CD8+ T-cells in mice.

IL-10:

Sasaki *et al.*, 2004,[119] had reported that IL-10 deficient mice display increased susceptibility to *P. gingivalis*-induced periodontal bone loss, further supporting a role for IL-10 in the control of destructive inflammation. **Sasaki *et al.*, 2008,**[120] had reported that mice with an additional deficiency of IL-12p40, *i.e.*, IL-10 deficient and IL-12p40 deficient mice, are rendered resistant to periodontal bone loss in the same experimental model, and so are T-cell/IL-10 double-deficient mice. However, since IL-12p40 is shared between IL-12p70 and IL-23, it is uncertain whether Th1 or Th17 cells mediate periodontal destructive effects in this hyper inflammatory model of IL-10 deficiency.

In fact, a role for Th1 cells in inflammatory bone resorption has been ruled out, in rheumatoid arthritis, where they actually inhibit osteoclastogenesis (**Sato *et al.*, 2006**).[121] There is a balance of pro and anti-inflammatory cytokines, and may be more important for protection against infection-induced destructive inflammation than a strong polarized response on either side.

This concept is supported by **Alayan *et al.*, 2007,**[5] in another study they found that deficiencies in either Th1 (IL-12, IFN-γ) or Th2 cytokines (IL-4) resulted in periodontitis.

The periodontal health represents a state where, the activity of pro-inflammatory/antimicrobial cytokines which controls infection is balanced by anti-inflammatory mechanisms to prevent unwanted inflammation. In contrast, when this balance is disrupted, either due to pathogens that dysregulate host defense mechanisms or genetic differences in host immunity, disease progression and tissue destruction may occur.

In a recent review by **Gemmell *et al.*, 2007,**[41] it was hypothesized that the role of T-cells in periodontal disease may not be one of protection or destruction, but rather that T-cells play a homeostatic role in maintaining a balance between host and the biofilm.

Romagnani, 2006,[112] have hypothesized that would include the participation of CD4+CD25+ regulatory T-cells (Tregs), which suppress Th1 and Th2 responses, although their effects on Th17 cells are largely unknown .In contrast to a balanced state, **Stashenko *et al.*, 2007**[134] had reported**,** strongly polarized response in any direction may be detrimental to the host.

Kawai *et al.*, 2000,[72] in his experimental studies have shown that gingival injection of rats with *A. actinomycetemcomitans* 29-kDa outer membrane protein and LPS, followed by adoptive transfer of antigen-specific Th1 clone cells, leads to high levels of bone resorption. Whereas these studies underscore the potential for destructive inflammation in strongly biased Th1 conditions, they do not necessarily rule out Th1-mediated protection under more balanced, physiological conditions.

Interleukin 1 (IL-1):

Dinarello, 1987,[28] have reported that IL-1 is a principal mediator of inflammatory responses acting on many cell types other than those of the immune system and is itself produced by many different cells, including macrophages, endothelial cells, B cells, fibroblasts, epithelial cells, and osteoblasts in response to microorganisms, bacterial endotoxin or exotoxins, complement components, or tissue injury.

Dinarello, 1987 et al,[28] in his studies isolated the cDNA clones, that have shown two forms, IL-1 alpha and IL- 1 beta, both of which bind to the same receptor and have identical biological properties. IL-1 has a central role in T cell activation resulting in transcription of various genes, including IL-2 and CD25 (IL-2 receptors), and the activated T cell then secretes a number of cytokines that affect the cells in the immune response (**Janeway, 1989**).[63]

IL-1 appears to potentiate proliferation and/or differentiation of mitogen or antigen activated B cells rather than have a direct effect, as it does on T cells so that IL-1 may act by priming B cells to respond to subsequent activation (**Freedman et al., 1988**).[34]

Dewhirst et al., 1985,[27] have reported that IL-1 plays an important role in bone resorption, which is a feature of chronic inflammatory diseases such as periodontal disease and rheumatoid arthritis.

Matsushima et al., 1985,[86] have stated that B cells, which are present in increased numbers in the progressive periodontal lesion, have been shown to produce IL-1 and therefore may be an important factor in the bone loss that occurs in this disease.

Interleukin 2 (IL-2):

IL-2 was first discovered through its activity as a T cell growth factor. It is secreted and synthesized by T cells after activation by antigen or mitogen and plays an essential role in T cell division. **O'Garra, 1989,**[99] reviewed that IL-2 act also as an autocrine growth factor, it also induces T cell cytotoxicity and natural killer (NK) cell activity.

Miyawaki et al., 1987,[89] had reported that IL-2 also acts as a growth factor for activated human and murine B-blast cells by inducing increased Ig secretion.

Interferon Gamma (IFN-Gamma):

Morris et al., 1988; O'Garra, 1989,[91,99] have reported that IFN-gamma is produced during an immune response by antigen-specific T cells and NK cells recruited by IL-2. Its regulatory effects include the activation of macrophages to enhance their phagocytosis and tumor-killing capability as well as activation and growth enhancement of CTL and NK cells.

Snapper and Paul, 1987; Finkelman et al., 1988,[133,32] have stated that IFN-gamma plays a major role in the control of immunoglobulin isotypes produced during an immune response. In the mouse IgG2a is enhanced with suppression of other IgG isotypes and IgE. **Finkelman et al., 1988,**[32] have stated that IgG2a is very effective at fixing complement and promoting NK cell-mediated killing so that IFN-gamma promotes the ability of the humoral immune system to destroy microbial pathogens.

O'Garra, 1989,[99] reviewed that IFN-gamma inhibits most of the activities induced by IL-4, suggesting that these two cytokines reciprocally regulate Ig isotype production in T-cell dependent immune responses. **Scott, 1993**

reviewed,[124] the presence of IFN-gamma appears to be a requisite for the induction of the Thl subset.

Interleukin 4 (IL-4):

Sher et al., 1992,[128] reviewed that, IL-4 produced by T cells, mast cells, and basophils and is an important factor in the clonal expansion of antigen-specific B cells. This IL-4 enhances the synthesis of IgE in both the mouse (**Coffman et al., 1986**)[20] and human (**Del Prete et al., 1988**),[26] IgGl in murine B cells (**Coffman et al., 1986**),[20] and IgG4 in human cells (**Lundgren et al., 1989**).[84] Therefore, it would appear that IL-4 modulates humoral responses to different antigenic stimuli. IL-4 contributes to negative immune regulation by its ability to reduce IL-2 receptors and thus inhibiting some IL-2-induced activities. These include the IL-2-induced generation of NK cells and IFN-gamma-enhanced activities, including the activation of macrophages and their antimicrobial activity (reviewed in **O'Garra, 1989**).[99]

IL-4 can also block macrophage nitric oxide generation necessary for the killing of intracellular parasites (reviewed in **Modlin and Nutman, 1993**).[90]

In the current review, it is interesting to note that treatment of mice with either IL-4 or anti-1L-4 antibodies has demonstrated that the differentiation of Th0 clones toward a Th2 phenotype is dependent on the presence of IL-4 (**Scott, 1993**)[124]

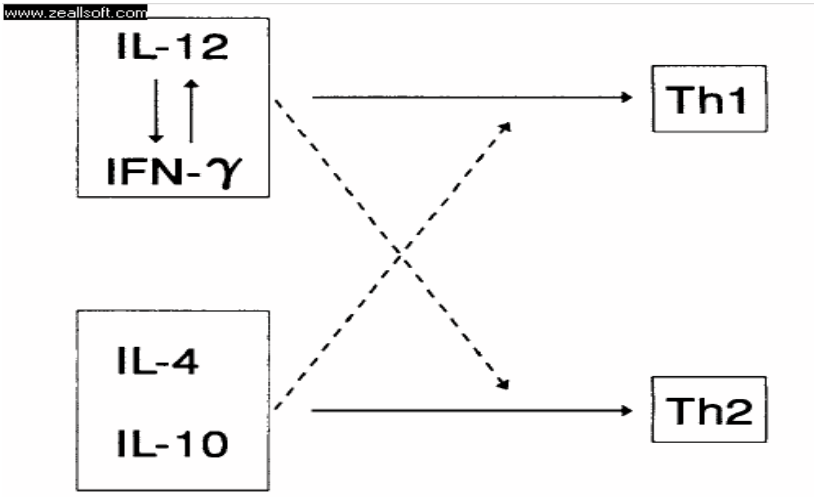


FIGURE 2. IL-12 and IL-4 regulate Th1 and Th2 T cell generation. Th1 and Th2 responses are maintained by positive (→) and negative (- - →) feedback mechanisms (Trinchieri, 1993).

Interleukin 5 (IL-5) :

IL-5 has several effects on B cells, including the induction of IgM and IgA production. **O'Garra, 1989**,[99] had stated that this cytokine is chemotactic for and is a potent stimulus for the growth and differentiation of eosinophils.

Interleukin 6 (IL-6):

O'Garra, 1989,[99] reviewed that IL-6 is produced by both hemopoietic and nonhemopoietic cells and induces immunoglobulin secretion in both preactivated murine and human B cells and therefore induces the final maturation of B cells into high rate Ig-secreting cells. IL-6 activity is not

restricted to B cells and appears to be a key member of the cytokine network being expressed at high levels by many cell types both constitutively or after activation and interacts with many targets (**Tovey et al., 1988**).[152] Like IL-1, it appears to have a major role in the mediation of inflammatory and immune responses initiated by infection or injury. IL-6 activates T and B cells, stimulates hemopoietic, and both IL- I and IL-6 seem to mediate most of the effects ascribed to macrophages in lymphocyte activation (**Van Snick and Nordon, 1990**)[158]

Th1/Th2 cells in periodontal disease:

Several studies have discussed the role of Th1 and Th2 cells and their cytokine profiles in periodontal disease. Over the past decade, it has been proposed that as stable periodontal lesions resemble a delayed type hypersensitivity lesion and progressive lesion involves large number of B cells, these lesions may be mediated by Th1 and Th2 cells respectively.

It has been shown that a strong innate response leads to a Th1 response under the influence of IL-12, IL-2, IFN-γ, while a weak innate response leads to a Th2 response under the influence of IL-4 cytokines. In a stable lesion, IFN-γ enhances the Phagocytic activity of both neutrophils and macrophages and hence contains the infection. In case of a poor innate immune response and minimal Il-12 production, a weak Th1 response may not contain infection.

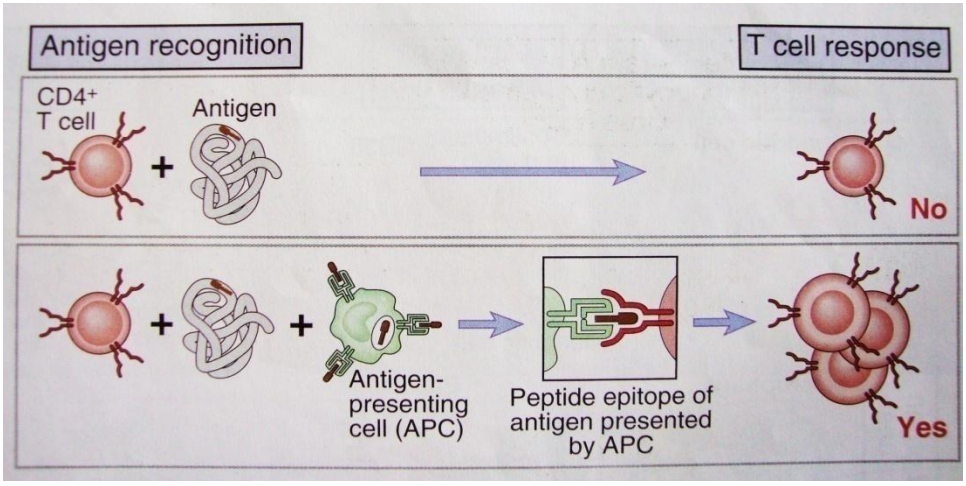
Mast cell stimulation and the subsequent production of IL-4 would encourage a Th2 response, B cell activation and antibody production. If these antibodies are protective and clear infection, the disease will not progress but if they are not protective, as in case of IgG2, the lesion will persist. Continued B-cell activation may result in large amounts of IL-1 and hence tissue destruction.

Despite its simplicity, the Th1/Th2 model does not adequately explain many findings with respect to T-cell mediated immune responses. Various studies have reported discrepancies with regard to predominance of Th1 or Th2 response or the involvement of both Th1 and Th2 cells in diseased tissue.

Antigen recognition, Processing and Presentation:

About 95% of T cells in peripheral blood express a TCR comprising a αβ chain; the remaining T cells express a γδ TCR. CD4 and CD8 molecules act as co receptors with TCR. Each T cell has a unique receptor generated by recombination of a variable (V), diversity (D), and joining (J) gene segments during thymic maturation. This random process generates many non functional receptors, which die by a process called positive selection. Self reactive T cells are eliminated by a process called negative selection,

which is one of the mechanisms by which immune system is made tolerant and does not attack self cells.



Yamazaki K, Nakajima T etal 2004,[163] has stated that T cells do not recognise whole antigen. Proteins derived from infectious organisms are taken up by antigen presenting cells processed into antigen peptides and presented on cell surface by binding to a peptide binding groove of MHC.

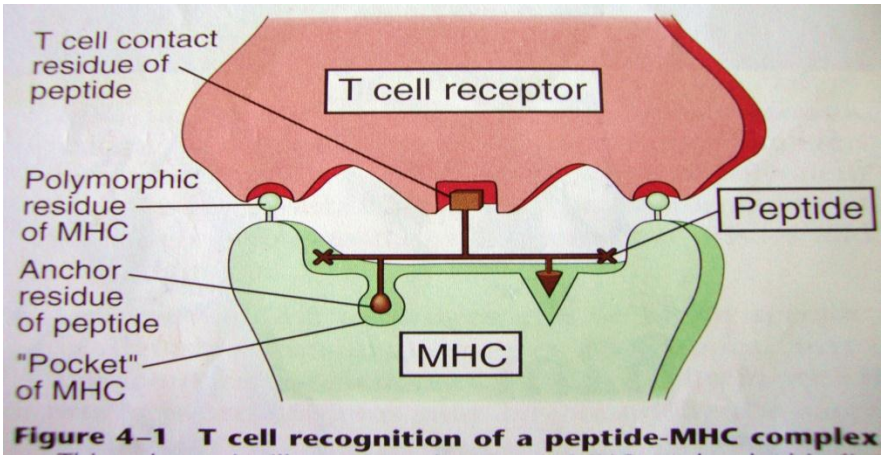


Figure 4–1 T cell recognition of a peptide-MHC complex

Antigen processing is divided into 2 pathways which lead to peptide fragments being presented by MHC class I or Class II molecules. Proteins that are derived within the cells such as those produced during viral or intracellular bacterial infections are processed by endogenous pathway. Proteins derived from outside the cell such as antigens phagocytosed by macrophages and dendritic cells are processed by exogenous pathway.

King CA, Wills MR et al 2005,[76] has stated that all cells in body are capable of processing and presenting antigens via MHC class I pathway. Only professional Antigen presenting cells bear MHC class II and present antigens to CD4+ T cells.

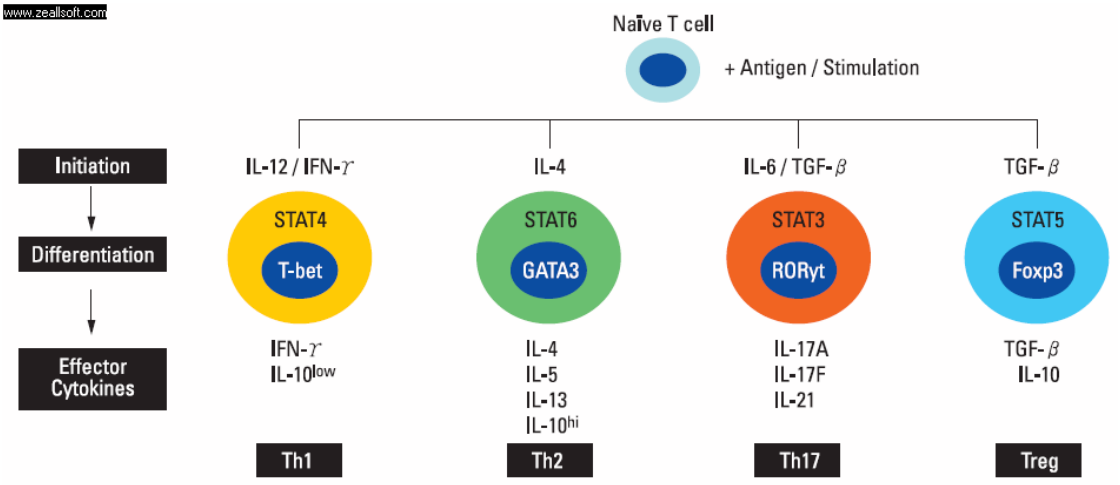
Yamazaki K, Nakajima T.et al[163] have stated that non peptide antigen is recognised by CD1 expression on antigen presenting cells- monocytes, B cells and dendritic cells. Group 1 CD1 (a, b, c) are expressed by professional APCs.

Amanuma R, Nakajima T et al 2006 [6] have stated that CD1d is expressed by monocytes, macrophages, B cells.CD1d presents self lipids to specific T cells called NK T cells implicated in regulation of autoimmune response.

T cell Subsets:

T cells are functionally divided into T helper, regulatory and cytotoxic cells. Helper and suppressor cells help to regulate immune response by releasing a variety of regulatory mediators or cytokines. Cytotoxic cells are able to kill other cells through cell-cell interactions. Cells of the innate response influence T cell differentiation by influencing the cytokine milieu in tissues or by draining lymph nodes where antigen specific T cells expand in response to antigen presentation.

Ansel KM, Lee DU, Rao.A et al [8] upon antigenic stimulation, naïve CD4 + T cells can be differentiated into diverse T helper cell subsets like Th1, Th2, Th17 and Treg. From *in vitro* and *in vivo* studies, instructive cytokines and transcription factors which are specific for each effector lineage were identified. In the early initiation stage, the unique signal transducer and activator of transcription (STAT) are activated by the environmental cytokine signal which leads to induction of lineage master regulators. Transcription factors from each distinct subset activate and control the various downstream genes and this mechanism is further enhanced and stabilizes the lineage commitment with epigenetic modification by specific stimuli and the action of transcription factors. As a result, effector cytokines and modulators can be released from each CD4 + T helper cell subsets and these further regulate immune responses accordingly to antigens.



Powrie and Coffman, 1993,[108] have stated that a successful immune response to an infectious agent is dependent on activation of appropriate effector functions.

Kelso, 1990,[74] documented that extracellular pathogens are generally eliminated by antibody binding that facilitates complement fixation, phagocytosis, and activation of the Fcγ-dependent release of reactive oxygen species by monocytes/macrophages and polymorphonuclear neutrophils (PMN) .

Elimination of intracellular pathogens (viruses and certain bacteria) depends on the destruction of the infected cell by antigen-specific cytolytic T cells (CTL) and activated macrophages (**Kelso, 1990**).[74]

Inactivation of other organisms occurs by the release of enzyme-rich granules from eosinophils and mast cells as in the case of helminths,

intracellular lysis in the case of infection of macrophages by bacterial pathogens .(**Kelso, 1990**).[74]

Bloom et al., 1992,[18] had shown the evidence that human CD4+ T cells have cytokine patterns and functions comparable to those that exist in mice.

Romagnani, 1992,[115] has stated that Thl cytokines are generally elevated in successful responses to many intracellular pathogens, while Th2 cytokines are elevated in allergic diseases and helminths infections. Th2 cells stimulate mast cells, eosinophils and IgE antibodies. Th1 cells increase the ability of macrophages to kill intracellular and extracellular pathogens and also mediate delayed type hypersensitivity (DTH) reactions. Th1 and Th2 cells play different roles not only in protection against exogenous antigens but also in immunopathology. Therefore, each T helper subset induces and regulates effector functions targeted at different antigens and pathogens.
Although most studies have been on CD4+ cells, it has been reported that the majority of CD8+ T cells also have a Thl-like profile.

Street and Mosmann, 1991,[93] have been reviewed that these T cells are cytotoxic and although the pattern of secretion is Thl, IL-2 secretion is low or undetectable. **Bloom et al.1992,**[18] have stated that IFN-gamma is secreted at about the same level as Th1 clones, whereas most other

cytokines are secreted at lower levels. There is evidence that Th2-like CD8+ T cells also occur in both the human and mouse.

Recently, a report by **Inoue et al., 1993**,[59] shown that cytolytic CD8+ T cells could not suppress CD4+ T cells, whereas suppressor CD8+ T cells could suppress the proliferative responses of both Th1 and Th2 CD4+ cells by the production of IL-10. It is now evident that the pattern of cytokine production by both CD4+ and CD8+ T cells appears to be related to the function of the cell rather than to phenotype and **Bloom et al.(1992)**[18] have proposed the general concept of type 1 and type 2 T cell subsets (Figure)

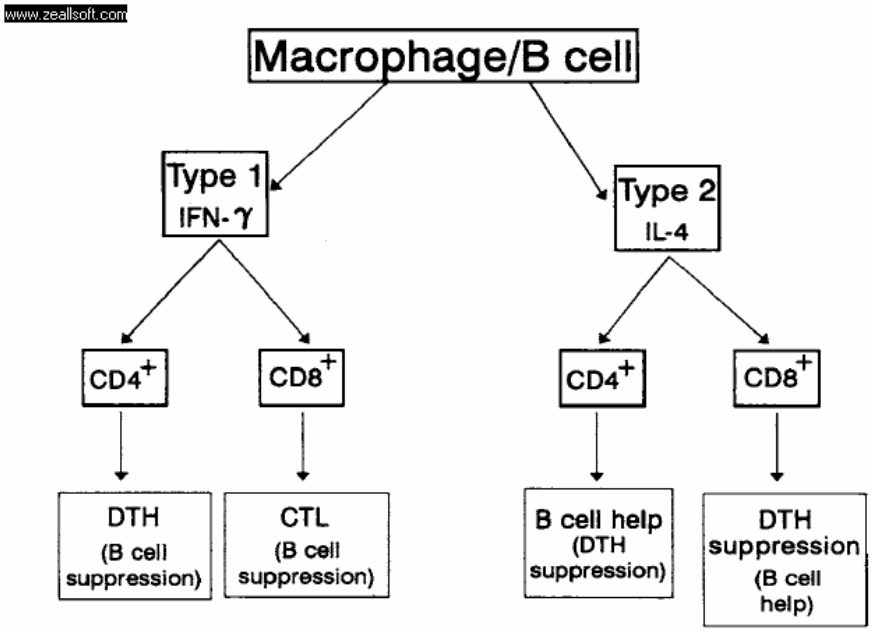


FIGURE 1. Relationship of T cell subsets with function (Bloom *et al.*, 1992).

Scott and Kaufmann, 1991,[122] had reported that the division of CD4+ T cells into distinct subsets based on cytokine production could represent a major advance in understanding immune responses, particularly those

associated with infectious disease. **Mosmann, 1991,**[93] have reviewed that most resting T cells secrete IL-2 when first stimulated and then differentiate into cells secreting a number of cytokines.

Mosmann, 1991,[93] have stated that Th1 cells produce IL-2 and IFN-gamma, whereas Th2 cells secrete IL-4, IL-5, and IL-6 after activation by antigen or mitogen. **de Waal malefyt et al., 1992,**[25] have stated that both types of T cells produce IL-10 &1L-13.

de Waal Malefyt et al., 1992,[25] had reported a third category of Th cell clones can produce all of these cytokines and have been designated Th0 cells. It has been established that murine IL-2 and IL-4 producing clones can be derived from the same cells, suggesting that cytokine phenotype is acquired during T cell differentiation and is not secondary to the expansion of distinct subpopulations predetermined to produce a specific cytokine pattern.

Modlin and Nutman,1993,[90] believed that on activation, antigen-specific naive CD45+ T cells progress into a Th0 cell and then differentiate further into Thl or Th2 cells with repeated antigen stimulation. **Mosmann, 1991,**[93] have stated that this differentiation of cells secreting distinct cytokine patterns is assumed to be under the influence of signals from APC and

cytokines acting in both paracrine and autocrine fashions during antigen stimulation.

Mosmann, 1991,[93] have stated that several secretion patterns seen in short term in vitro clones may represent early stages in the differentiation pathway and the Thl and Th2 cytokine profiles are assumed to represent final chronically stimulated helper T cells. Strong antiparasitic responses are often either Thl orTh2 in nature as are long-term T cell clones.

Trinchieri, 1993,[154] have shown that the use of cytokines and anti-cytokine antibodies in animal experiments has confirmed that once a Th1 or Th2 type of response is determined early during an immune response, it remains stable unless major changes take place in the balance of cytokine production during the response.

Street and Mosmann, 1991,[93] had stated the functions of Th1 and Th2 CD4+ T cell subsets correlate with the cytokines they secrete. One of the major functions of helper T cells is to provide help for B cells to proliferate and differentiate into antibody secreting cells following exposure to antigen. This ability depends on the concentration of cytokines at the time of T-B cell interaction. Low levels of IFN-gamma produced by Thl cells induces antibody with an IgG2a isotypes, but high levels of this cytokine will have

an inhibitory effect on B cell responses. The result of Thl interaction can therefore be help or suppression of antibody production.

Street and Mosmann, 1991, reviewed that Th2 clones provide help for the formation of IgM, IgGI (mouse) or IgG4 (human), IgA, and IgE because of the production of the B cell proliferation and differentiation factors, IL-4, IL-5, and IL-6. **Coffman and Carty, 1986,**[20] have stated that IFN-gamma, on the other hand, inhibits the IL-4-induced IgE response.

Street and Mosmann, 1991, in vivo studies using cytokines and anti-cytokine antibodies have supported these observations providing support for the concept that the functional abilities of the Thl and Th2 subsets are mainly due to the cytokines they produce.

Recent studies by **Cox and Liew, 1992**,[22] suggest that the immune response to infection is in fact regulated by the balance between Th 1 and Th2 cytokines. These two pathways are often mutually exclusive, the one resulting in protection and the other in progression of the disease.

Recent studies by **Modlin and Nutman, 1993,**[90] have been focused on the cytokines that are responsible for immunity on the one hand or pathology on the other. The net effect of the Thl cytokines IL-2 and INF-gamma is to enhance cell-mediated responses, while that of the Th2 cytokine IL-4 is to suppress cell-mediated responses and hence enhance the resistance associated with immunity.

T cell activation:

First signal is specific requiring TCR recognising and binding to antigen bound MHC presented by antigen presenting cells. Second signal is non-specific (co-stimulatory molecules), If T cells receive these 2 signals, they will undergo proliferation, differentiation and then acquire effector function

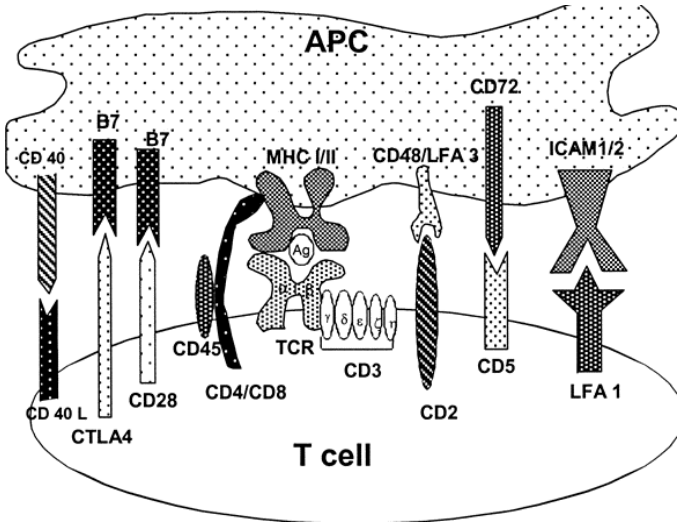


Fig : Antigen presentation and T cell activation

Co stimulatory molecules belong to Ig super family, TNFR family and integrin family. CD28 co stimulation in T cells augments production of IL-2, promote cell cycle progression, induce effector cytokine production, suppress cell death by altering bcl-2, caspase protein function and enhance memory cell development.

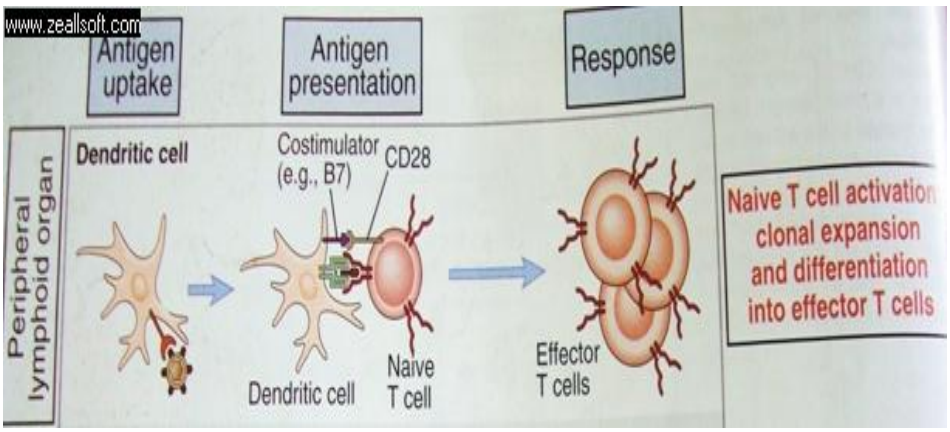


Fig : co-stimulators for T- Cell activation

Antigen presenting cells express co stimulatory molecules such as CD80, CD86, and CD40 which will engage T cells and amplify TCR signalling through their corresponding receptors CD28, CD40L. Binding of CTLA4 to B7 molecules plays an essential role in limiting the actively proliferating responses of activated T cells to antigens and B7 on surface of APCs.

Th1/Th2 PARADIGM:

O'Garra and Vieira, 2007,[97] had reviewed that when a naïve CD4+ T-cell is exposed to antigen, IL-12 derived from macrophages or dendritic cells, drives it to develop into a Th1 phenotype, characterized by the secretion of IFNγ. Other cytokines, such as IL-2, lymphotoxin (TNFβ), and IL-10, have also been called Th1 cytokines, but none defines this lineage as clearly.

Glimcher, 2007,[49] have stated that The Th1 cell expresses the IL-12 receptor subunit IL-12Rβ2, which further commits a cell to proceed along this differentiation program. This process is dependent on the transcription factor STAT-4, which is activated by IL-12, and also on T-bet, which is considered the master regulator of the Th1 lineage.

Conversely, when a newly activated Th cell is exposed to IL-4, differentiation to a Th2 phenotype occurs. Th2 cells secrete IL-4, IL-5, and IL-13, which are co-regulated transcriptionally.

Yamashita *et al.*, 2007, had stated that Th2 development is dependent on the transcription factors STAT-6, GATA-3, and c - maf. Th1 and Th2 development is mutually antagonistic and self-reinforcing, in part because IL-4 and IFN-γ antagonize one another at cellular and molecular levels.

Th1/Th2 skewing in periodontitis:

Balance of the two helper subsets determines what kind of adaptive immune response is utilised to eliminate infection. Periodontal tissue destruction is strongly associated with both Th1 and Th2 cytokine expression. Th1 effectors produce inflammatory cytokines that can mediate tissue destruction, anti-inflammatory cytokines produced by Th2 cells are involved in tissue homeostasis during inflammatory destruction and subsequent repair or remodelling**(Teng Y.T)**[147] CD4+ T cells participate in

tissue inflammatory responses and alveolar bone destruction; through the cytokines they produce. **(Teng Y.T)**[148]

Some studies reported that cytokine profiles of periodontitis patients were predominantly of Th1 type. But, other studies have stated that Th2 cytokine profile is expressed in periodontitis (**Hourihaddad Y et al)**[57]

Th1 cytokine profile is seen in early or stable lesion and Th2 cells are involved in advanced or progressive lesions. Production of IFN γ by Th1 cells enhances phagocytic activity of both neutrophils and macrophages and hence containment of infection. However, lesion persists due to continual formation of plaque biofilm. If innate response is poor, low levels of IL12 produced, poor Th1 response may occur, which may not contain the infection.

Reinhardt RA et al (1988)[110] evaluated lymphocyte subsets within gingival biopsies and in peripheral blood samples from the same patients using direct immunofluorescence. The T helper/T suppressor cell ratio did not vary significantly between blood and any gingival tissue disease group or location, but a trend toward lower relative numbers of Th cells in the sulcular infiltrates of active sites was noted.

Wassenar A et al (1995)[160] cloned and characterised T lymphocytes isolated from gingival tissue obtained from 4 periodontitis patients and tested their proliferative responses to whole cell antigens of

Porphyromonas gingivalis, Prevotella intermedia, Aggregatibacter Actinomycetemcomitans, human collagen type I, and bacterial Hsp and for the production of cytokines by flow cytometry analysis. He proposed that collagen specific CD4+ Th2 like T cells contribute to the chronicity of periodontitis but their modes of activation might be controlled by Th0 like T cells specific for periodontitis associated bacteria.

Yamazaki K, Nakajima T, Hara K. (1995)[162] showed that IL 4 is the prominent cytokine in periodontitis affected tissues. Immunoregulation of both periodontitis and gingivitis are T cell dependent, but in periodontitis Th2 cells predominate and thereby control B cell activation.

According to **Fujihashi et al (1996)**,[36] when gingival mononuclear cells (GMC) were isolated from inflamed tissues of adult periodontitis examined by flow cytometry, 20-30% of lymphocytes were CD4+ T cells. Although human CD4+ T cells are capable of producing an array of Thl- and Th2-type cytokines, the CD4+ T cells associated with periodontitis are limited to production of IFN γ, IL-6, IL-13 and IL-10.

Gemmell E, Yamazaki K, Seymour GJ. (2002)[47] showed that P. *gingivalis*-specific T-cells produced both Th1 and Th2 cytokines, regardless of the APC population.

Gemmell E and Seymour GJ (2004)[45] stated that mast cell stimulation and production of IL4 would encourage a Th2 type response, B cell activation

and antibody production. If the antibodies are protective and clear infection, disease will not progress, but if they are non protective, lesion will persist and continued B cell activation would result in large amount of IL1 and hence tissue destruction

Ito H et al (2005)[61] established T cell clones from the gingival tissues of 19 periodontitis patients and examined their gene expression. All the gingival T-cell clones expressed mRNA for TGF-β1, CTLA-4, and CD25, and all the T-cell clones from peripheral blood expressed IFN-γ and TGF- β1 mRNAs.

CD8+ cells:

Naïve CD8+ cells express a number of cell surface receptors and ligands that enable them to home to secondary lymph nodes. The environment created in these lymph nodes allows rare antigen specific CD8+ cells to be activated. Differentiation involves expression and storage of large amount of enzymes. Modification of cell surface receptors occurs; allowing migration from lymph nodes to circulation and eventually into inflamed tissues, where they exert their effector function. Cytotoxicity is induced by perforin/granzyme pathway or Fas/FasL pathway. Tc1 cells secrete type I cytokines IL-2, TNF α and β, IFN γ. Tc2 cells secrete IL-4, 5,6,10

In periodontal tissues, CTLs can be found next to the periodontal fibroblasts in deep gingival tissues, where direct cytopathic and degenerative changes occur. There is said to be a Tc2 like cytokine expression in periodontitis patients.

These cells do not directly participate in periodontal tissue destruction; however they play some protective roles in fighting periopathogens by generating important cytokines for both innate and adaptive immune response and by cytolytic killing of bacterial infected or damaged tissue of cells.

Celenligil H et al (1990)[19] analysed the phenotypic properties of gingival lymphocytes in 12 adult periodontitis patients. CD4+ and CD8+ cells were evenly distributed within these infiltrates.

Th1/Th2 reciprocity:

The two Th1/Th2 subsets exert a suppressive effect on each other through a variety of mechanisms. Their signature cytokines, IL-4 and IFN-γ suppress each other, i.e., IFN suppress Th2 responses while IL-4 production down regulates Th1 responses. The intra cellular signals are also capable of similar responses. For example, GATA- 3 suppresses STAT- 4 and thereby Th1 responses, while STAT-5 reduces T-bet expression. T-bet by it, can down regulate GATA- 3 expression.

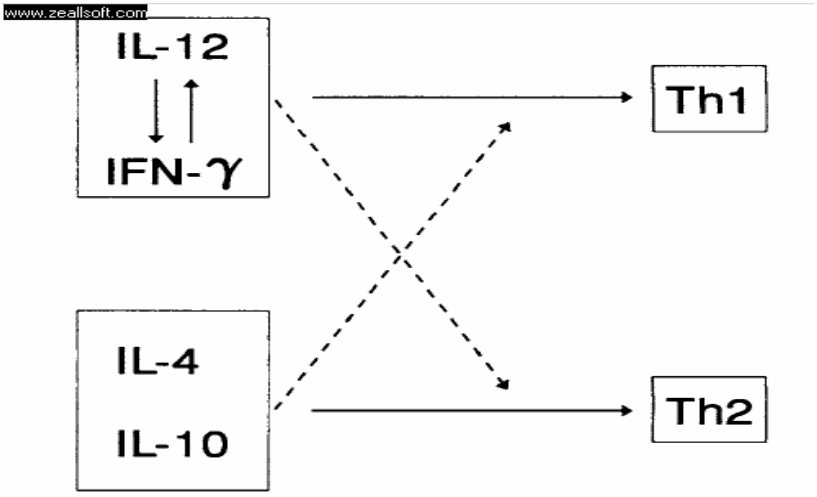


FIGURE 2. IL-12 and IL-4 regulate Th1 and Th2 T cell generation. Th1 and Th2 responses are maintained by positive (→) and negative (- - →) feedback mechanisms (Trinchieri, 1993).

Th1 CELLS:

Brandtzaeg P, Kraus FW, have stated that Th1 cells are believed to afford protection against the intracellular pathogens. These cells produce the interferons (IFN) that are involved in macrophage activation, which in turn plays an important role in phagocytosis, complement fixation and opsonisation.

Development

Th1 development requires inductive signals that initiate a network of intercommunicating intracellular events that culminates in the differentiation of the naive T cell to a differentiated Th1 cell.

Inductive signals :

Buduneli N, Bicakci N, Keskinogˇlu, have reported that Th1 polarisation is induced by the cytokines **IL-12 and IFN-α,** both of which are present in the inflammatory milieu. These cytokines are normally produced by the dendritic cells or the natural killer (NK) cells. In the periodontal environment, dendritic cell infiltration has been strongly associated with gingival inflammation. These cells have toll like receptors (TLR) on their surface that enable them to respond to the pathogen-associated molecular patterns (PAMPs)-lipopolysaccharide (LPS), fimbriae etc of the periodontopathogens. NK cells have also been identified in the inflamed periodontium and are thought to respond to a variety of stimuli, including lipid antigens such as lipotechoic acid, in a TLR independent, CD 1 dependant manner.

Intracellular signalling pathway:

IFN-α/ IFN-γ in conjunction with IL-12 activates signal transducer and activator of transcription (STAT) - 1 in the naive CD4 T cells. Activated STAT-1 upregulates the master regulator of Th1 differentiation – T box expressed in T cell (T- bet). Activation of T- bet leads to induction of the receptor IL-12Rβ_2, making these cells more responsive to IL-12. This in turn, causes activation of STAT-4, which upregulates IFN-γ production by the Th1 cells. At the later stages of differentiation, IL-18Rα receptor expression is up regulated, thereby, making these cells responsive to IL-18.

Concomitant action of IL- 12 and IL- 18 leads to the full repertoire of IFN-γ production that is characteristic of the effector function of Th1 cells.

Gately et al., 1998,[40] in experimental studies, had reported that IL-12, acting via the Stat4 signaling pathway, is the primary inducer of Th1 development. Mice deficient in IL-12, IL-12 receptor or Stat4 have markedly reduced Th1 responses

O'Garra, 1998,[98] in experimental studies, had reported that while mice lacking IL-18 or IFNγ have defective in vivoTh1 responses, these cytokines do not directly induce Th1 differentiation but rather augment IL-12-induced Th1 development.

In contrast to Th2 differentiation, very little is known about the molecular basis of Th1 differentiation.

Kaplan et al,1996,[70] hypothesised that the only known transcription factors whose absence results in failure to generate Th1 cells are STAT4 & IRF 1.

Lohoff et al,1997,[83] documented that in the absence of either of these (STAT4 & IRF 1) will not result in the generation of Th1 cells.

Th2 CELLS:

Th2 cells are thought to have evolved as protection against parasitic helminths. These cells produce IL-4 in addition to IL-5 and IL-13; cytokines that are involved in immunoglobulin (Ig) class switching in B cells. The importance of Ig-G that is produced as a result of this class switching in periodontal disease pathogenesis has been well documented.

Development

Inductive signals:

Th2 development is induced by production of IL-4, which in turn is produced by the naive T cells or the mast cells/ macrophages**.** In the periodontal milieu, naive T cells constitute only a small proportion T cell infiltration within the tissues; it is comprised predominantly of the memory or the effector cells. Therefore, they are unlikely to be the major source of IL- 4 in the gingiva. On the other hand, both macrophages and mast cells have been identified in the periodontal environment and as early responders; they are the likely source of IL-4 production.

Intracellular signalling:

IL-4 signalling results in activation of STAT-6 which then up regulates the master switch of Th2 differentiation, GATA-binding protein-3 (GATA- 3). In addition to GATA-3, STAT- 5 signalling is also required for complete Th2 differentiation**.** GATA-3 auto-amplifies its own gene, subsequent

activation is, therefore, not dependant on other signals. Consequently, Th2 cells are thought to be a more stable phenotype.

Nelms et al., 1999,[96] in experimental studies has shown that the critical Th2-inducing cytokine is IL-4, which mediates its effects on naive Thp cells through the Stat6 signaling pathway. Mice lacking either IL-4, IL-4 receptors, or Stat6 fail to develop Th2 cells.

Seder and Paul, 1994,[125] has reported that Th1 and Th2 cytokines both promotes the growth/differentiation of their subset and inhibit the growth/differentiation of the opposing subset. For example, IL-4 produced by Th2 cells acts in a positive manner to activate IL-4 receptors present on naive Thp cells to initiate the Th2 differentiation process.

Hodge et al 1996,[56] stated that the Th2 specific transcription factor, c-maf, along with NFAT & NFAT interacting protein confers on a non-Tell the ability to produce IL-4.

Kim et al 1999,[75] stated that mice with c-maf deficient have impaired IL-4 production.

Zhang et al 1997,[171] hypothesized that GATA-3 like c-maf is Th-2 specific and upregulates during Th-2 differentiation.

Zhung, Flavell et al 1997, Ouyang et al 1998,[171,103] stated that GATA-3 appears to regulate a broad spectrum of Th-2 cytokine genes.

Agarwal and Rao et al 1998,[3] hypothesized that along with GATA-3, STAT-6 is also necessary for chromatin remodelling of IL-4/IL-13 locus found to occur in developing Th-2 cell. Thus GATA-3 and STAT-6 have more global influence on Th-2 cytokine gene expression and mediates this effect by directly altering the chromatin structure of Th-2 specific gene loci.

Th1 and Th2 differentiation:

In 1989, Mosmann and Coffman,[94] reported that CD4+ T helper cells could be divided based on their cytokine profile. The Th1 cells produce IL-2, IFN-γ and their development is regulated by the transcription factors T-bet and STAT-4. Th2 cells are regulated by STAT-6, c-maf and GATA-3 and they produce cytokines such as IL-4, IL-5, IL-9 and IL-13. Hyper activation of Th1 and Th2 type responses results in over production of cytokines leading to auto-immune disorders and allergic diseases respectively. The presence of T-regulatory cells in both scenarios prevents diseases manifestations by suppressing their activity.

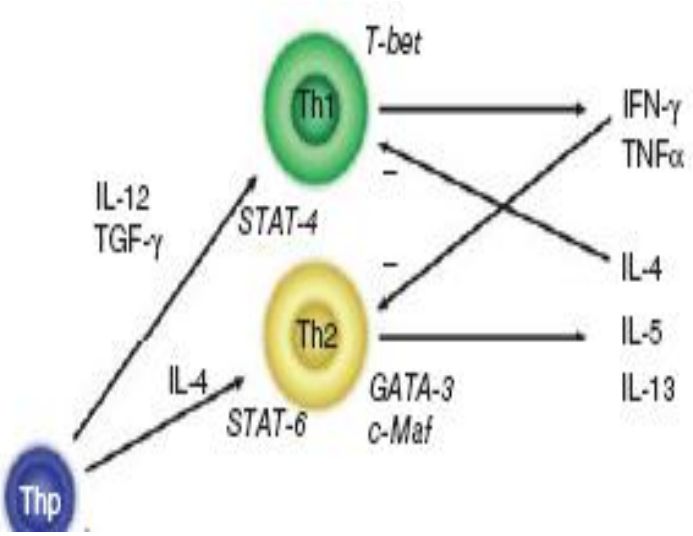


Fig: Th differentiation

Th1 cells produce IL-2 which induces strong proliferation of T cells, they promote B cells to produce IgG2a isotype antibody.Th1 mediates activation of macrophages through IFN γ. Th1 secreted IFN γ is mainly responsible for pro inflammatory effects of macrophages and cytotoxic T cells. IL12, IFN γ induce Th1 development.

Th2 After activation in secondary lymph nodes Th2 cells are retained in T cell areas of B cell germinal centres, where they exert their helping effects by direct contact with antigen presenting B cells and production of cytokines. IL 4, 13 induces B cell class switching. IL 5 activates eosinophils. IL6, IL10 inhibit generation of Th1 cells, reinforcing bias to Th2 response. Th2 cells stimulate high titres of antibody production. IL4, 6 required for Th2 development

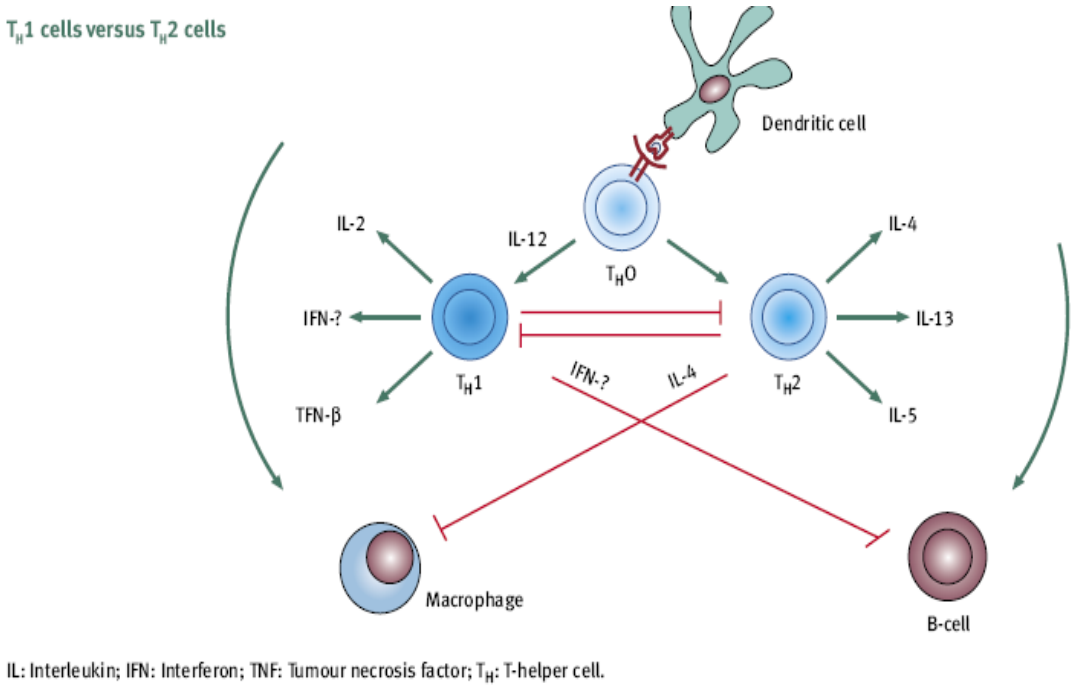


Fig : Th1 and Th2 cells

Th1 differentiation:

T-bet:

Szabo et al., 2000,[141] have stated that T-bet, also known as Tbx21 belongs to the T-box family of transcription factors and is the only known T-box gene specifically expressed in the lymphoid system. T-bet is rapidly and specifically induced in developing Th1 but not Th2 cells, **Lighvani et al., 2001,**[80] have stated that T-bet expression appears to be controlled by both TCR and IFN-γR-STAT1 signals, **Lighvani et al., 2001; Mullen et al.,**

2001; Afkarian et al., 2002,[80,2] have reported that not by the IL-12-STAT4 pathway.

Mullen et al., 2002,[95] have stated that T-bet expression seems to be self regulated; retrovirally expressed T-bet induced T-bet in Th2 cells, **Afkarian et al., 2002,**[2] have stated that it is dependent on STAT1, likely through IFN-γ signaling. Indeed, IFN-γR-STAT1 signaling maintains high-level of T-bet expression in developing Th1 cells. **Afkarian et al., 2002,**[2] have stated that IFN-γ promotes Th1 commitment through T-bet.

Szabo et al., 2000,[141] in vitro studies have shown that retrovirus-mediated over-expression of T-bet in differentiating or fully differentiated Th2 cells resulted in an induction of IFNγ expression, accompanied by a reduction of IL-5 and, to a lesser extent, of IL-4. Such an effect of T-bet is cell-intrinsic because IL-4 and IL-5 expression were unchanged in control Th2 cells that co-existed in the same culture.

Afkarian et al., 2002,[2] in experimental studies have shown that the strength of TCR signaling appeared to be important in quantitatively affecting T-bet expression levels and thus Th1 differentiation. In addition, T-bet-deficient CD4 T cells failed to produce IFN-γ in response to TCR stimulation, even under Th1-polarizing conditions. Therefore, T-bet is critically involved in initiating Th1 development through both inducing the Th1 program and repressing the opposing Th2 program.

Hlx:

Hlx is a Th1-specific homeobox gene that interacts specifically with T-bet. **Mullen et al., 2002,**[95] have shown that its expression becomes detectable 3 days after Th1 differentiation and is restricted to Th1 but not Th2 T cell clones. Hlx appears to be a target gene for T-bet because retrovirus-mediated expression of T-bet and a dominant negative form of T-bet induced and inhibited Hlx expression, respectively. **Mullen et al., 2002,**[95] have stated that Hlx and T-bet synergistically promote IFN-g expression when co-expressed. Thus, as a cofactor for T-bet, Hlx appears to enhance the activities of T-bet and thus Th1 differentiation. Further study is needed to investigate whether Hlx promotes or is required for optimal Th1 responses under physiological conditions.

STAT4:

Ouyang et al., 1999; Yang et al., 1999,[102,164] have stated that STAT4 is critical for IL-12 signaling and also for differentiation of Th1 cells. The mechanism of STAT4-promoted IFN-γ expression is largely unknown.

Xu et al., 1996 have stated that STAT4 may act through binding to regulatory genomic regions of the IFN-γ gene or through interacting with general transcriptional co-activators p300/CBP (**Bhattacharya et al., 1996; Zhang et**

al., 1996; Korzus et al., 1998).[17,77] Thus STAT4 is critical for amplifying IFN-γ production, but not the initial IFN-γ expression.

Th2 differentiation:

GATA-3:

GATA-3 is a member of the GATA family of transcription factors. While it was previously shown to be critical for embryo development, **Zheng and Flavell, 1997,**[174] have shown that GATA-3 dictates Th2 differentiation by regulating Th2 cytokine production through binding to the IL-4 locus that encompasses the IL-4, IL-5 and IL-13 genes. Forced expression of GATA-3 in Th1 cells led to the induction of IL-4 production (**Zheng and Flavell, 1997**).[174] Th2 differentiation and responses are abolished in vitro and in vivo when GATA-3 is deficient in T cells. **Zhu et al., 2004,**[178] have shown that even in fully differentiated Th2 cells, when GATA-3 is blocked then IL-5 and IL-13 production will be affected but not IL-4 production ,due to the fact that GATA-3-binding sites were found in the promoters of IL-5 and IL-13 but not in IL-4.

STAT6:

In vitro studies by **Shimoda et al.,1996; Takeda et al., 1996,**[131,142] have shown that STAT6, activated by IL-4 stimulation, is the major signal transducer in IL-4-mediated Th2 differentiation. However, in vivo studies by

Finkelman et al., 2000; Jankovic et al., 2000; Min et al., 2004,[33,65,88] have shown that Th2 responses can be obtained independently of STAT6.

Kurata et al., 1999; Zhu et al., 2001,[78,176] in vitro studies have shown that one of the mechanisms of STAT6 is to promote Th2 differentiation through inducing high levels of the transcription factorGATA-3. STAT6 activities were found necessary and sufficient for inducing GATA-3.

c-Maf, IRF-4:

c-Maf is an AP-1 family transcription factor selectively up-regulated in Th2 cells. **Kim et al., 1999,**[75] have stated that c-Maf is required for the production of IL-4 but not other Th2 cytokines.

Lohoff et al., 2002; Rengarajan et al., 2002,[82,111] have stated that IRF-4 is also shown to be required for Th2 cell differentiation. IRF-4-deficient cells produce much less IL-4, but this defect can be rescued by over-expression of GATA-3, suggesting that IRF-4 promotes Th2 differentiation through up-regulating GATA-3.

In vivo studies by **Nicolas Dutzan, Jorge Gamonal et al,**[181] have shown that, in active periodontal lesions, T bet has been increased to about 2.8fold when

compared to GATA-3. Whereas IFN-γ was the highest over expressed cytokine that was detected in active periodontal lesion.

In vivo studies by **Ohyama et al in 2009,**[101,] have stated that IFNγ did not vary significantly after treatment. They also discovered that there was no significant difference in expression levels of IFNγ between periodontal lesions and control sites.

Lester et al 2007,[80] have shown that the gingival concentration of IFNγ was significantly greater at moderate CAL sites than at normal-slight CAL sites, and less than normal level in severe CAL sites. In active periodontal lesions, IFNγ and T-bet was significantly over expressed compared with inactive lesions. They came to conclusion that IFNγ might play a destructive role in the pathogenesis of periodontal disease.

De Heens et al,2009,[24] have stated that the level of IL-4 increased significantly after non surgical periodontal treatment. Some studies suggested that IL-4 and Th-2 biased response are protective against periodontitis progression. The high levels of alveolar bone loss in mice induced by Porphyromonas gingivalis were inversely correlated with the Th-2 response to this bacterium, but others suggest the opposite. An elevated Th-2 profile in smokers of periodontitis patients may constitute a risk for conversion of

periodontal stability into progressive disease. Based on this study they consider Th-2 cells and Th-2 related cytokines might play a protective role in the periodontitis progression.

Materials and methods:

Recruitment of samples into the study:

Age and gender matched subjects were recruited from the outpatient department of Ragas Dental College and Hospital, Chennai. A written consent by the patient to participate in the study will be obtained. The study has been approved by our Institutional Review Board. The age of subjects in both the healthy and diseased groups was ranging from 35 to 55 years.

Selection criteria:

Group A: healthy subjects (control group) who had PD <3mm with no clinical attachment loss and BOP <10%

Group B: Advanced chronic periodontitis patient (Test group) who had PD ≥ 8 mm, clinical attachment loss ≥ 5mm and bleeding on probing, atleast one or more teeth indicated for extraction.

Healthy tissue samples were obtained from patients who had reported to the department for crown lengthening or during impacted/IIIrd molar removal

Diseased tissue samples from patients indicated for extraction in advanced/severe periodontitis.

The dimension of the tissue sample: 3mm × 2mm.

Exclusion criteria:

- Patients with history of periodontal therapy or antibiotic therapy in the past 6 months
- Patients with history of systemic diseases that may affect the periodontal status
- Pregnancy and Lactation
- Smokers

Homogenization of tissue samples:

Tissue samples were completely minced and homogenized in a homogenizer using TRY reagent.

The obtained tissue samples were thoroughly washed with PBS (Phosphate buffered saline) and stored in RNA later (RNAase free solution) and stored in -80°C until further RNA extraction is carried out.

Total RNA extraction:

Isolation of total RNA:

Total RNA was isolated from health and diseased tissue by using total RNA isolation reagent (Trisol, Medox) kit.

Principle:

Single step guanidium acid phenol method emphasizes on the ability of guanidium isothiocyanate (GITC) to lyse cells, denature protein and inactivate intracellular ribonuclease rapidly. The presence of β-mercaptoethanol in the mixture enhances the solubilization properties of the GITC extraction buffer. Acid phenol extraction (pH<5.0) selectively retains cellular DNA in the organic phase and aids in the extraction of proteins and lipids. The addition of chloroform further removes lipids and establishes two distinct phases, an organic phase containing the DNA, proteins and lipids and an aqueous phase containing the RNA.

Reagents:

1. Trisol kit has the following components: Phenol, guanidium isothiocyanate, urea, detergents, buffering agents and stabilizers.

2. Chloroform (Molecular biology grade).

3. Isopropanol (Molecular biology grade).

4.75% ethanol (Analytical grade) – to 7.5 ml of absolute ethanol 2.5 ml of autoclaved deionised water was added.

Procedure:

1ml Trisol was added to control and treated cells and swirled gently for 15 min and then kept at 4°C for 5 min to permit complete dissociation of

nucleoprotein complexes. To this 0.2 ml chloroform was added, shaken vigorously for 15 sec and placed at 4°C for 5 min. The lysate was then centrifuged at 12,000 rpm for 15 min at 4°C, which yielded, lower organic phase containing DNA and proteins and upper aqueous phase containing RNA. The volume of the aqueous phase was about 40-50% of the total volume of the lysate.

The aqueous phase was carefully transferred to a fresh eppendorf micro centrifuge tube without disturbing the interphase. Equal volume of isopropanol was added, mixed and kept at 4°C for 10 min. It was again centrifuged at 12,000 rpm for 15 min at 4°C to precipitate the RNA.

The supernatant was removed and the pellet was washed with 75% ethanol and air-dried. The RNA pellet was then dissolved in 30µl of sterile deionised water and placed in water bath at 60°C for 10 min to ensure maximum solubility of RNA. The RNA sample was subsequently vortexed gently and quantified before storing at – 80°C.

Quantification of RNA:

Diluted RNA sample was quantified spectrophotometrically by measuring the absorbance (A) at 260 nm. An absorbance of 1 is equivalent to RNA concentration of 40µg/ml. Therefore, the yield can be calculated by multiplying the absorbance at 260 nm with dilution factor of 40. The purity of

the RNA was assessed by determining the absorbance of the sample at 260 nm and 280 nm. The purity of RNA obtained was around 1.8.

Quantitative Real Time-PCR Analysis of T–bet & GATA-3 Expression:

Principle

Real time-PCR selectively amplifies the first strand of cDNA that has been synthesized in vitro by RNA polymerase from mRNA templates by reverse transcription. The cDNA is first denatured by heating in the presence of a large molar of two oligonucleotide primers and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerases. The cycle of denaturation, annealing and DNA synthesis is then repeated many times. Real-time experiments monitor and report the accumulation of PCR product, as detected by increased fluorescence, during thermal cycling. Data collection during the early exponential phase of PCR allows the software to accurately calculate initial template quantities.

First strands cDNA synthesis:

Kit Components:

- Omniscript reverse transcriptase (RT - enzyme)
- RNAase inhibitor (10 U/µl)
- Oligo - dT primer (10 µl)

- dNTP - Mix (5 mM each dNTP)
- 10× Buffer RT
- RNase - free water

Quantitative Real time PCR - SYBR® Green I Assay kit (Qiagen,Germany)

Kit Components

- **2X Reaction buffer (7.5ml):** qPCR Master Mix Plus for SYBR® Green I dNTP containing dNTPs, Hot Goldstar DNA polymerase, $MgCl_2$ (5mM final concentration), SYBR® Green I, stabilizers and passive reference.
- **50 mM $MgCl_2$ (1.5 ml).**
- The resulting cDNAs from RT reaction were diluted with Milli-Q water (1:10). Real Time-PCR was carried out on **Mx 3000p Multiplex quantitative PCR system (Stratagene).** Reaction was performed using 5.0 µl of 2X reaction buffer, 1µl of 100 nM of both forward and reverse primers of gene of interest, 1 µl of forward and reverse primers of 18S rRNA, 3.0 µl of diluted cDNA was made up to a 10µl reaction. The thermal cycling protocol was as follows: 95^{o}C for 5 minutes, followed by 40 cycles of PCR at 95^{o}C for 30 seconds and 58^{o}C for 30 seconds. All reactions were performed in triplicate along with no template control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 50ºC - 95ºC for each sample to determine the presence of

multiple amplicons, nonspecific products, and contaminants.. The relative amount of mRNA was calculated by using the comparative Ct method.

- All the reactions described above were carried out in triplicate.

T-bet :

T-bet forward: TCC AAG TTT AAT CAG CAC CAG A

T-bet reverse: TGA CAG GAA TGG GAA CAT CC

GATA-3:

GATA forward: ATC ATT AAG CCC AAG CGA AG

GATA reverse: TCT GAC AGT TCG CAC AGG AC

Once the amplification was generated at the end of the reaction, the corresponding ct (cycle threshold) values were obtained.

Data interpretation:

The values obtained in the Real time-RT PCR was interpreted using the formula of fold change, $2^{-\Delta\Delta ct}$ = [ct of gene of interest – ct of internal control] of disease sample – [ct of gene of interest – ct of internal control] of health sample.[180]

This form of equation may be used to compare the gene expression in two different samples (health and disease) or (treated and untreated). Each sample is related to an internal control gene.

Results:

Gingival tissue samples were obtained from 10 patients each from periodontally healthy and diseased groups and total RNA isolated and extracted. The mRNA expression for T-bet and GATA-3 was evaluated by using Real time RT-PCR.

The present study results showed the expressions of T-bet and GATA-3 are summerarized in the following table 1.

T-bet	**Group**	**Mean + SD**	**18sRNA value**	**Delta CT value**	**Fold Change**
	Disease	25.45±0.02	14.22±0.00	11.23±0.02	1.12±0.02
	Health	30.94±0.03	18.59±0.00	12.35±0.03	0.00±0.03
GATA-3	**Group**	**Mean + SD**	**18sRNA value**	**Delta CT value**	**Fold Change**
	Disease	28.79±0.09	14.22±0.00	14.58±0.09	-4.39±0.09
	Health	28.76±0.06	18.59±0.00	10.18±0.06	0.00±0.05

T-bet:

The results reveals that the mean ct value of the health group is 30.94±0.03 and for the diseased group is 25.45±0.02. The Delta CT value derived from

the formula, [$2^{-\Delta\Delta ct}$ = (ct of gene of interest – ct of internal control) of disease sample – (ct of gene of interest – ct of internal control) of health sample] is 12.35±0.03 for health group and 11.23±0.02 for diseased group. This shows that the amplification has occurred in fewer cycles in disease than in health. This is represented as fold change from health to disease. An increase of 1.12±0.02 fold change was observed in disease when compared to health.

	Mean+SD	-	internal control	=	Delta ct	
$2^{-\Delta\Delta ct}$ =	25.45	-	14.22	=	11.23	(disease sample)
	30.94	-	18.59	=	12.35	(health sample)

Fold change = 11.23 - 12.35 = 1.12

GATA-3:

Similarly in GATA-3 results reveals that the mean ct value of health group is 28.76±0.06 and diseased groups 28.79±0.03. The Delta CT values derived from the formula, [$2^{-\Delta\Delta ct}$ = (ct of gene of interest – ct of internal control) of disease sample – (ct of gene of interest – ct of internal control) of health sample] is 10.18±0.06 for healthy group and 14.58±0.09 for diseased group. This shows that the amplification has occurred later than that of health, with decreased expression in disease group than in health group. This is represented

as fold change from health to disease. A decrease of 4.39 fold was observed in disease group when compared to health.

	Mean+SD	-	internal control	=	Delta ct	
$2^{-\Delta\Delta ct}$ =	28.79	-	14.22	=	14.58	(disease sample)
	28.76	-	18.59	=	10.18	(health sample)

Fold change = 14.58 - 10.18 = 4.39

Discussion:

It is well established that the onset/ progression of periodontal disease is largely determined by the nature of the inflammatory response to the challenge put forth by the plaque/biofilm.[149] The central role played by the T cells in the pathogenesis of periodontal disease, was established in early 1940.[106] .

However, with a greater understanding of their biological functions, T cells are now believed to be involved in the homeostasis of periodontal tissues,[41] modulation of the inflammatory/immune responses[12] and mediation of the bone loss observed in periodontal disease.[150]

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. The presence of two different subsets of Th cells-Th1 and Th2 was described by Parish and Liew.[106] Later, Mossman and Coffman delineated two distinct cytokine profiles associated with the Th1 and Th2 cells.[92]

While the CD8+ cells may be involved in killing and removing bacteria infected damaged host cells, they do not directly mediate tissue destruction. The CD4+ T cells are directly involved in the inflammatory response observed in periodontal disease, through mediation of both hard and soft tissue destruction, primarily through the production of cytokines.[47]

In periodontal disease, cytokines are central to the pathogenic process involved in disease activity. It has been postulated that "appropriate"

cytokine production results in protective immunity, while "inappropriate" cytokine production leads to tissue destruction and disease progression.[42]

It has been established that the cytokines expressed by these cells contribute to the state of protective verses destructive phases of disease progression. Based on their cytokine expression profiles, the CD4+ T helper cells have been classified into 2 distinct subsets namely, Th1 &Th2.[94] Th1 cells differentially produce IL-2& IFN-γ, while Th 2 cells produce IL-4, IL-5& IL-6. The two subsets exert a suppressive effect on each other, through a variety of mechanisms.

Earlier studies have postulated a role for Th1 &Th2 subsets in periodontal disease.[45] The pathogenesis of periodontitis was classically reviewed in terms of Th1/Th2 paradigm where the Th1 cytokines were associated with early stage of lesions, while the destructive Th2 cytokines in the advanced lesion.[126]

Irrespective of the predominant Th subset present in progressive lesions, the cytokine milieu is thought to be important for both differentiation and functioning of the Th profile.[15]

Two sets of cytokines are intimately associated with the Th subsets- inducers and effectors. The inducing cytokines are responsible for establishing the lineage largely to the activation of the transcription factors. It is known that master transcription factor T-bet along with

STAT1 drives towards Th1 differentiation,[138,69] while GATA3, STAT 6 & C-maf drive towards Th 2 differentiation .[178,142]

The transcriptional regulation of the Th cell differentiation in periodontal disease still remains an area of concern. With the emerging concepts on the plasticity of the Th cells the role of the transcription factors have gained more relevance today, than never before.[8] Hence the aim of this study was to evaluate the transcriptional regulation of Th1 &Th2 subsets in periodontal health and disease.

Gingival tissue samples obtained from periodontally healthy individuals and from patients with advanced periodontal disease have been included in the study. Healthy tissue samples were obtained from patients who reported for crown lengthening or for third molar extraction. The diseased tissue samples were from sites of advanced periodontal disease, as indicated by probing depth > 8mm, attachment loss > 5mm and radiographic evidence of greater than 2/3rds of bone loss, and hence indicated for extraction.

The tissue samples were carried in an RNAase inhibitor containing solution and homogenized immediately. Cryopreservation was not done to prevent any degradation of proteins. Total RNA was obtained from the homogenates using total RNA extraction kit. Real time – Reverse transcription PCR was then carried out with SYBR green containing

master mix kit to evaluate the mRNA expression of T-bet and GATA-3 in periodontal health and disease.

The results of our study showed a marginal, insignificant increase in the levels of T-bet in periodontal disease when compared to health, while that of GATA-3 had shown a significant decrease in periodontal disease, when compared to health.

It would appear that these results suggest a minor insignificant increase of the Th1 cell and more profound down regulation of Th2 in advanced periodontal lesions. These results seem to suggest that advanced lesions are more likely to be Th1, rather than Th2 dominated.

Th1 dominated lesions are thought to be associated with increased production of IFN-γ, which is its signature effector cytokine. IFN-γ is closely associated with macrophage activation.contineous production of IFN-γ May therefore lead to excessive presence of macrophage in chronic inflamed periodontal tissues. This could, in turn, leads to excessive osteoclastic activity, causing the bone loss that is a classical feature of progressive periodontal disease.

At the same time, a reduced Th2 activity could lead to the suppression of IL-4 and IL-10 production.IL-4 is known to be anti inflammatory cytokine that exerts significant effect on dampening of the inflammatory response. Further IL-10 is known to exert strong suppressive effect on osteoclastogenesis. [25]

The decrease in the level of these two important cytokines could, have led to the uncontrolled inflammatory response and bone loss observed in advanced periodontitis lesions.

The results of our study are in agreement with that of Fujihashi K, Yamamoto M,[35,36] et al who have suggested similar roles for the Th1/Th2 cells in periodontitis. Previous literature also suggests that Th1and Th2 subsets have a counter balancing and regulatory effects on each other. These effects are exerted at both transcriptional and effector cytokine levels.

Recent evidence indicates that T-bet and GATA-3 have direct suppressive effects on each other. The primary effect in the lesion observed could therefore have been due to upregulation of T-bet. This upregulation could in itself have led the suppression of GATA-3. It could be hypothesized that although T-bet upregulation did not show much stastical significance, its influence on the progression of periodontal disease was profound.

These results must however be interpreted with caution. Transcription factors exert their influence on differentiating cells through binding on the promoter region of the DNA. However, the production of these transcription factors is influenced more by the inducing signals in the inflammatory milieu, than the state of differentiation of the Th cells. Consequently, T-bet production is directly related to the presence of IFN-α, IL-12, while GATA-3 is dependent on the IL-4 levels.

As the lesions in our study where end stage of periodontal disease, with active inflammation, the levels of the anti inflammatory IL-4 levels were likely to be decreased. Previous studies have shown decreased IL-4 levels in both mRNA in tissues and proteins in the inflammatory fluids (GCF and saliva) in progressive periodontal lesions. This decreased Il-4 production could have directly influenced in lowered GATA-3 expression observed in our samples.

The effect of these transcription factors are however not so straight forward. Both T-bet and GATA-3 are known to be the Master transcription factors that act on differentiating Th1 and Th2 cells respectively. Therefore T-bet acts primarily through binding to the promoter region of the IFN gene locus, while GATA acts through binding to the IL-4 gene locus. If the transcription factors were utilized by the differentiated cells, their tissue levels could presumably be depleted.

It could thus be argued that a decrease in the transcription factor levels may well be because of a greater establishment of a terminally differentiated Th cell lineage. In other words, a decrease in GATA could be because all the available GATA present were used up by the differentiated Th2 cells. This means that the tissue may, in fact be characterized by a preponderance of Th cells.

Seymour and co-workers,[127] have suggested that the early stable periodontal lesion was associated with a robust infiltration of Th1 cells. On the contrary,

other authors,[35,36] have proposed that a Th2 cytokine profile was associated with a stable lesion, while the progressive lesion was characterized by the Th1 profile.

A clearer picture could have emerged, had the effector cytokines been assessed simultaneously. In the absence of these cytokine profiles, it is difficult to interpret the results conclusively.

However, it is clear that an imbalance in the transcriptional activation of the Th1/Th2 cells is closely associated with chronic periodontitis lesions. A similar imbalance in the effector cytokine profile is known to influence disease activity.

A complete picture requires a concomitant study of the inducing, intra cellular and effector signals involved in Th functions.

The evaluation of the transcription factors should have been done in different stages of the disease process, viz in progression of gingivitis to periodontitis, early stages of periodontitis and in chronic lesions. This is one of the limitations of our study.

Similarly it would have been more appropriate to have evaluated the cytokine expressed by these two subsets to draw more meaningful conclusions. Also the simultaneous study of the newer subsets viz. the Th17, T reg and other Th subsets should be taken as the Th1/Th2 paradigm alone is insufficient in solving the many dilemmas that still

continue to linger in the pathogenic mechanisms that trigger the periodontal disease process.

Summary and conclusion:

The aim of the present study is to evaluate the transcriptional regulation of Th1 and Th2 subsets (T- bet and GATA-3), in Periodontal Health and Disease. 20 patients who attended the outpatient department of Ragas Dental College and Hospitals, Chennai were enrolled in the study. Patients were divided into two groups based on their periodontal health status- 10 Healthy and 10 Periodontitis patients. The gingival tissue biopsy samples were processed for real time reverse transcription polymerase chain reaction to detect the mRNA expression of T-bet and GATA-3 in periodontal health and disease samples.

The mRNA expression of the master transcription factor of Th1 subset, T-bet showed a marginal increase of about 1.31 fold. While the GATA-3 levels showed a 4.39 fold decrease from disease to health. It would appear that these results suggest an insignificant increase of the Th1 cell and more profound down regulation of Th2 in advanced periodontal lesions. These results indicate that advanced periodontal lesions are more likely to be dominated by Th1, rather than Th2 subsets.

Understanding the mechanism of transcriptional regulation of Th subsets in the pathogenesis of periodontal disease would result in the development of new therapeutic agents, aiming at these transcriptional factors which might be beneficial in suppressing the periodontal disease in future.

However further investigations are required to add credibility to this result of the present study.

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DEPARTMENT OF PERIODONTICS

1. NAME :
2. AGE & SEX :
3. OCCUPATION :
4. INCOME :
5. ADDRESS :

6. CONTACT NO :
7. BIOPSY :

    a) Type:

    b) Nature of specimen:

8. CLINICAL DETAILS:

    a) Site: b) Colour:

    c) Consistency: d) Size:

9. PROVISIONAL DIAGNOSIS:

10. PATIENT CONSENT:

I have been informed about the surgical procedure for removal of gingival tissue while extraction of my tooth/periodontal surgery that will not have any determined effect on my healing soft tissue. The tissue obtained will be used for research purpose only, the result which will be informed to my request. I hence forth give my consent for the above procedure.

DUTY DOCTOR PATIENT SIGN

HOD SIGN

Bar graph showing the fold change of mRNA expression of T-bet and GATA-3 in periodontal health and disease.

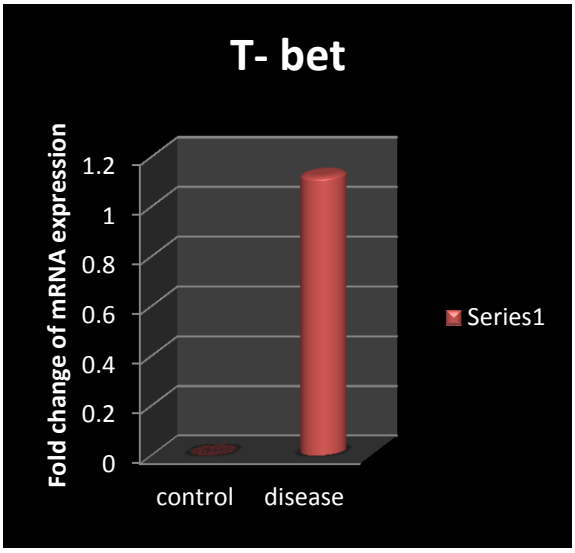
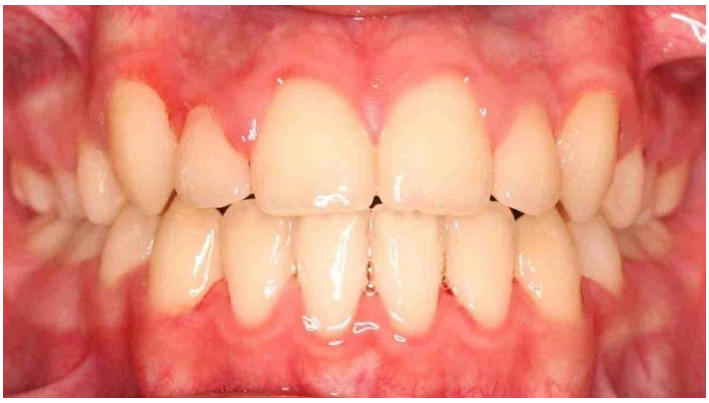


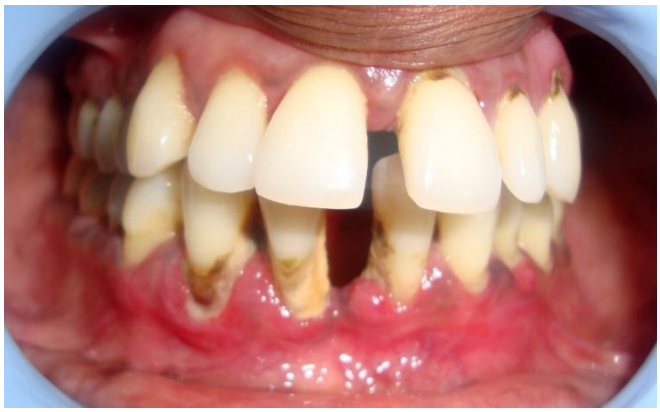
Table representing the fold change of mRNA expression of T-bet and GATA-3 in periodontal health and disease.

T-bet	**Group**	**Mean + SD**	**18sRNA value**	**Delta CT value**	**Fold Change**
	Disease	25.45±0.02	14.22±0.00	11.23±0.02	1.12±0.02
	Health	30.94±0.03	18.59±0.00	12.35±0.03	0.00±0.03
GATA-3	**Group**	Mean + SD	18sRNA value	Delta CT value	Fold Change
	Disease	28.79±0.09	14.22±0.00	14.58±0.09	4.39±0.09
	Health	28.76±0.06	18.59±0.00	10.18±0.06	0.00±0.05

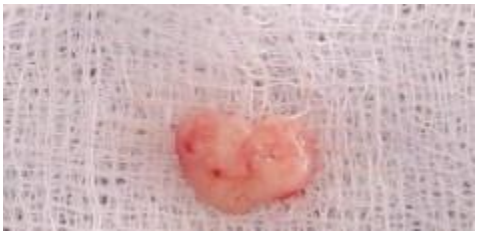
GROUP A HEALTHY GINGIVA



GROUP B PERIODONTITIS



Tissue Sample:

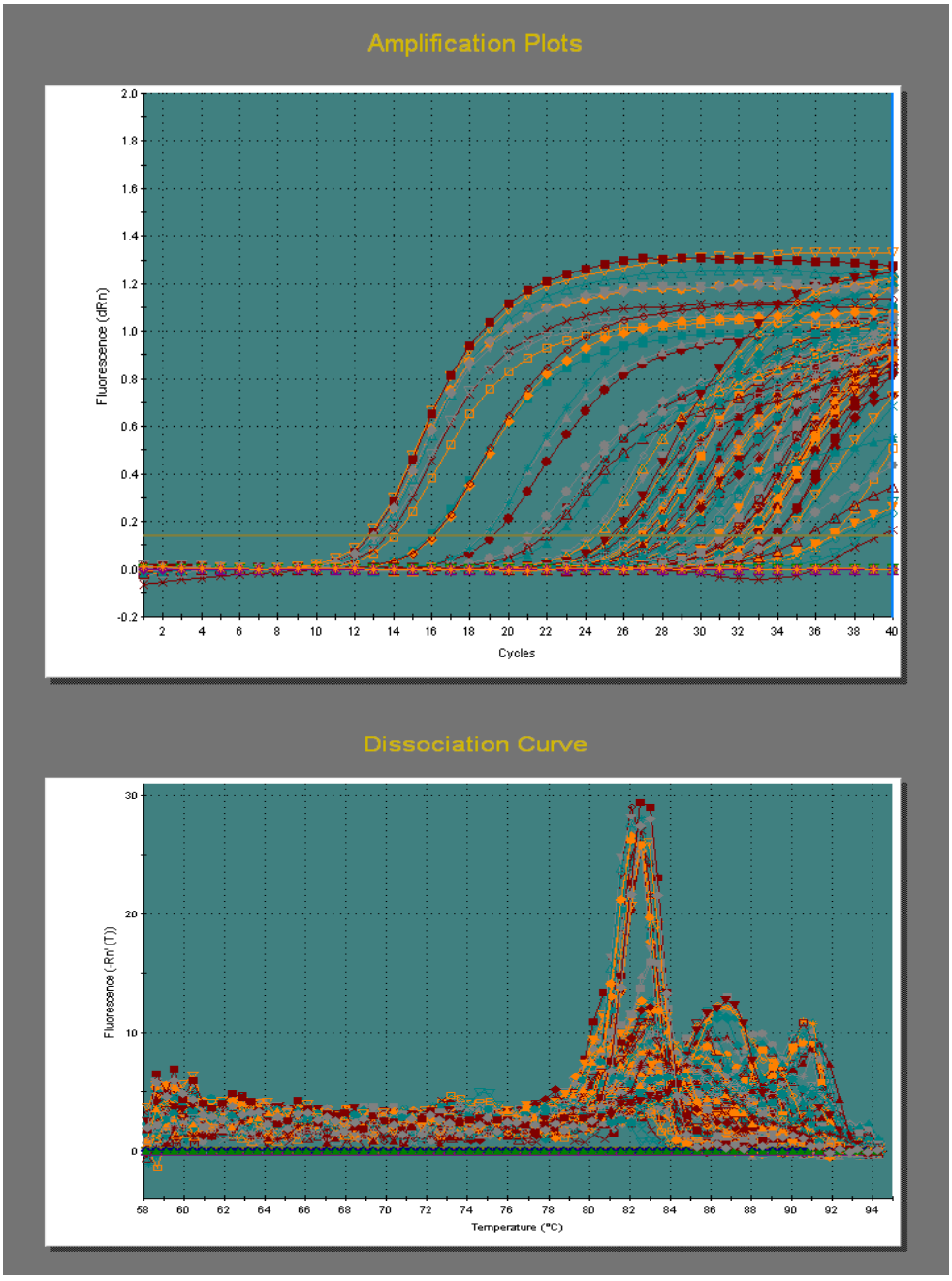


Centrifuge:

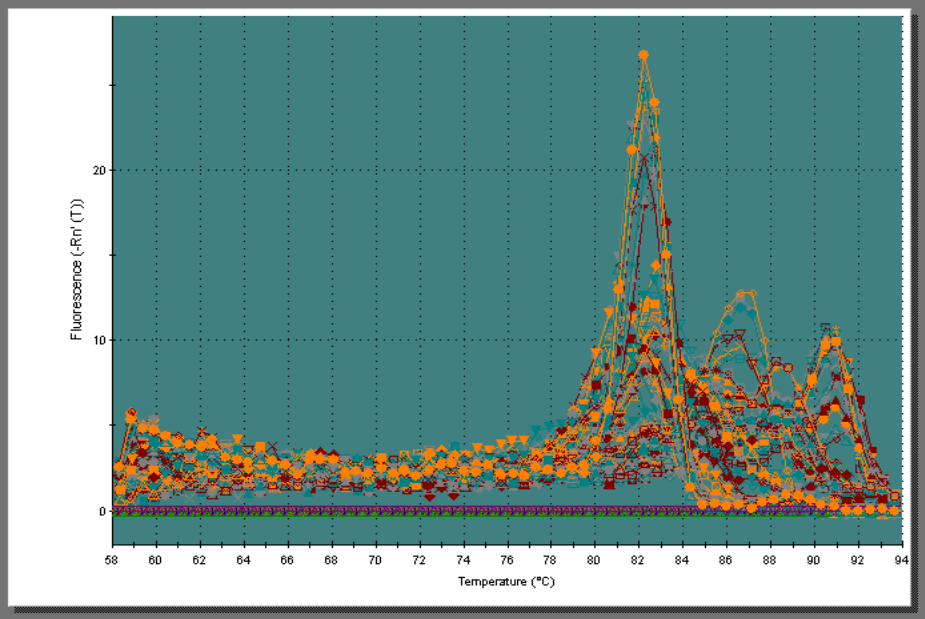
Mx 3000 p Real time PCR unit:

Mx 3000P Unit (inside view):

Amplification plots and Dissociation curve of T-bet and GATA-3.



Dissociation Curve



Amplification Plots