

Expression of toll like receptor 4 and cluster of differentiation 14 in chronic periodontitis patients- an immunohistochemical study

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Abstract

Introduction: Toll like receptors along with their co receptors contribute an essential task in immune and destruction mechanism. The aim of the present study was expression of Toll like receptor 4 and Cluster of differentiation 14 in healthy group and chronic periodontitis group.

Materials and Method: Forty gingival biopsies were harvested of which 10 were of healthy subjects and 30 from chronic periodontitis based on clinical records. All specimens were fixed, processed and stained by haematoxylin and eosin to histologically classify periodontitis. Later the sections were stained immunohistochemically using primary antibody for toll like receptor 4 and cluster of differentiation 14 respectively.

Results: The positive expression of both Toll Like Receptor 4 and Cluster of Differentiation 14 was defined as light or dark brown granules in the cytoplasm of the cell. Expression was higher in zone 1 followed by zone 2 and zone 3 in chronic periodontitis.

Conclusion: Toll Like Receptors act as a two-edged sword, not only maintaining periodontal health but also contributing to periodontal tissue destruction.

Keywords: TLR4, CD14, immunohistochemistry, Chronic periodontitis, Innate immunity.

Introduction

The Periodontal ecosystem is inimitable where, oral microorganisms are in constant contact with the host immune system. The tissue damage is initiated by the interaction between periodontopathogens and the host cells. The prime important challenge to the cells of the innate immune system like monocytes, macrophages and neutrophils, has been their difficulty to discriminate between receptors. It has been later realized that there exists a large number of receptors that recognize conserved patterns of pathogens called Toll like Receptors (TLR).⁽¹⁾ TLR signaling plays an important role in the innate immune response and maintenance of periodontal health. Nevertheless, over production of pro inflammatory cytokines due to chronic stimulation of TLRs lead to tissue destruction. The host immune system detects invading pathogens primarily through an array of pattern-recognition receptors which recognize conserved pathogen associated molecular patterns (PAMPs) through the lipopolysaccharide (LPS) of micro organisms.⁽²⁾

Toll-like receptors are essential mediators of innate antimicrobial and inflammatory responses and play important role in the adaptive immune response. Thus, when stimulated by certain agonists, toll-like receptors serve as adjuvant receptors that link innate and adaptive immunity.

TLR4 is a principal signaling receptor for gram-negative bacterial lipopolysaccharides (LPSs), which work synergistically with cluster of differentiation (CD)14, a glycosylphosphatidyl inositol-anchored membrane receptor.⁽³⁾

Cluster differentiation (CD) 14 is characterized by leucine-rich protein but it is distinct from TLRs in that it lacks typical transmembrane cytoplasmic domains. Mainly expressed on monocytes and macrophages, CD14 binds to LPS and mediates LPS-elicited cell signaling through TLR4.⁽⁴⁾ Expression of CD14 and TLR4 in periodontal tissues supports the importance of these receptors in periodontitis.

The present study are aimed at assessing immunohistochemically, the localization of TLR 4 and CD 14 in gingival tissues of healthy individuals and those with chronic periodontitis.

Materials and Method

A total of forty individuals which included 10 healthy and 30 chronic periodontitis patients based on Chi square test were considered for the study. The chronic periodontitis samples were further classified histologically into 3 groups i.e. mild, moderate and severe.⁽⁸⁾ The study was commenced after obtaining institutional ethical committee approval from institutional review board Maratha Mandal NGH institute of dental science and research centre. With reference id MDS 2012-13/1207. The nature and purpose of the study was explained to the subjects and written informed consent was taken. On the day of specimen collection, the periodontal parameters like bleeding indices (Ainomo and Bay), plaque index (Silness and Loe), gingival index (Loe and Sillness), probing depth and attachment loss were recorded using a UNC 15 periodontal probe. Periodontally healthy group constituted of individuals with no signs of

gingival inflammation, absence of bleeding on probing, probing depth < 3mm, no clinical attachment loss. The periodontitis group showed presence of gingival inflammation, probing depth \geq 5mm, clinical attachment loss > 3mm.

Patients with diabetes and other systemic illness, tobacco habitus, patients on any type of medication, pregnant and lactating mothers, patients who have undergone periodontal treatment within a period of 3 months were excluded from the study.

Method of specimen collection: The gingival tissue biopsy specimen was harvested from the tooth indicated for extraction. The teeth in healthy group were extracted for orthodontic reason. The gingival tissue was harvested from buccal or lingual site. All specimens were fixed in 10% formalin, followed by routine processing and embedding. Three sections each of the 4 μ m thickness were obtained. One section was stained with routine haematoxylin and eosin to histological classify periodontitis into three groups according to criteria given by Ukai et al.⁽⁸⁾ Two sections were taken in 3- Aminopropyl Triethoxysilane (APES) coated slide, stained for TLR4 and CD 14 respectively.

IHC Staining Protocol:⁽⁵⁾ For IHC staining, sections were placed on 3-aminopropyl triethoxysilane (APES)(A3648Sigma) coated slides. All reagents stored in refrigerator were brought to room temperature prior to immunostaining. The sections were maintained in a slide incubator at room temperature for all incubation. At no point in the entire staining procedure were the sections allowed to dry.

1. **Sectioning** – 1 sectioned each obtained from 3 serial sections of 4 μ m thickness were taken on silanized slides.
2. **Deparaffinization/ Rehydration**– sections were placed on slide warmer at 60°C for 40-45 minutes following which they were passed through 3 changes of xylene. Rehydration was done by passing the slides through 2 changes of 100% alcohol, 95% alcohol and 70% alcohol for 5 minutes each.

The slides were then washed in distilled water. Then the staining protocol was performed by using Thermo Ultravision Quanto detection system (TL-015-QHD). Primary antibody used was Rabbit polyclonal antibodies to TLR (Imgenex -578 A) at a dilution of 1:

90 in TBS. And for CD14 it was Ready to use Rabbit monoclonal antibodies to CD14 (Biogenex EPR3653).

Evaluation of TLR4 and CD14: Slides were visualized at 10X magnification under binocular light microscopes. Three zones were established according to criteria given by Ukai et al⁽⁶⁾ to evaluate the ratio of number of TLR4 and CD14- positive cells to total numbers of cells in connective tissues of each section. Zone 1 was tissue within 250 μ m of the basal cell layer of the pocket epithelium. Zone 3 was connective tissue within 250 μ m of the basal cell layer of the oral epithelium, and zone 2 was connective tissue between zones 1 and 3. The number of positive cells and the total number of cells per 250- μ m² field (unit area) of each zone was counted in three random fields of TLR 4 and CD 14 stained slides. The ratios of positive cells to total cells (positive cell ratios) were calculated in each group and each zone.⁽⁶⁾

Results

The positive expression of both TLR4 and CD14 was defined as light or dark brown granules in the cytoplasm of the cell. The comparison of TLR 4 and CD14 between chronic periodontitis and healthy groups was recognized and the histologic grading was compared in between the three zones.

TLR 4 and CD14 expression was statistically significant in all 3 zones of chronic periodontitis compared to healthy.(Fig. 1, 2, 3, 4).

Expression of TLR 4 and CD14 in chronic periodontitis group was higher in zone1 followed by zone 2 and zone 3. Positive correlation between TLR4 and CD14 was observed and was more evident in zone 1.

Comparison between the immunohistochemical localization of TLR4 and histologic grading, TLR4 was statistically significant in severe inflammatory group compared to mild and moderate inflammatory group. (Table 1 & Fig. 5)

The comparison between the immunohistochemical localization of CD14 and histologic grading, CD14 was statistically significant in moderate and severe inflammatory group compared to mild inflammatory group. (Table 2 & Fig. 6)

Table 1: Comparison of histological gradings in chronic periodontitis group with respect to TLR 4 scores at zone 1, zone 2, zone 3 and their average by Kruskal Wallis ANOVA

Histological grading	Zone 1		Zone 2		Zone 3		Average	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Mild	0.26	0.07	0.18	0.01	0.18	0.08	0.21	0.04
Moderate	0.28	0.06	0.26	0.07	0.26	0.07	0.27	0.06
Severe	0.39	0.10	0.39	0.13	0.30	0.07	0.36	0.08
P-value	0.0090*		0.0010*		0.0340*		0.0010*	
Pair wise comparison of histological gradings by Mann-Whitney U test								

Mild vs Moderate	p=0.4047	p=0.0161*	p=0.0710	p=0.0641
Mild vs Severe	p=0.0174*	p=0.0018*	p=0.0149*	p=0.0027*
Moderate vs Severe	p=0.0079*	p=0.0062*	p=0.1983	p=0.0048*

*p<0.05

Table 2: Comparison of histological gradings in chronic periodontitis groups with respect to CD 14 scores at zone 1, zone 2, zone 3 and their average by Kruskal Wallis ANOVA

Histological grading	Zone 1		Zone 2		Zone 3		Average	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Mild	0.22	0.10	0.16	0.07	0.20	0.04	0.19	0.07
Moderate	0.34	0.05	0.26	0.06	0.26	0.06	0.28	0.04
Severe	0.32	0.09	0.28	0.08	0.25	0.05	0.28	0.06
P-value	0.1000		0.0220*		0.1400		0.0490*	
Pair wise comparison of histological grading by Mann-Whitney U test								
Mild vs Moderate	p=0.0373*		p=0.0124*		p=0.0786		p=0.0332*	
Mild vs Severe	p=0.0699		p=0.0149*		p=0.0616		p=0.0174*	
Moderate vs Severe	p=0.9563		p=0.5112		p=0.9128		p=0.9782	

*p<0.05

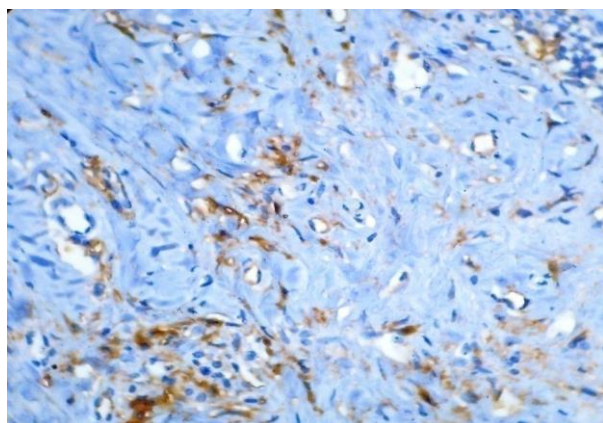


Fig. 1: Photomicrograph showing CD14 positivity in healthy group (40x)

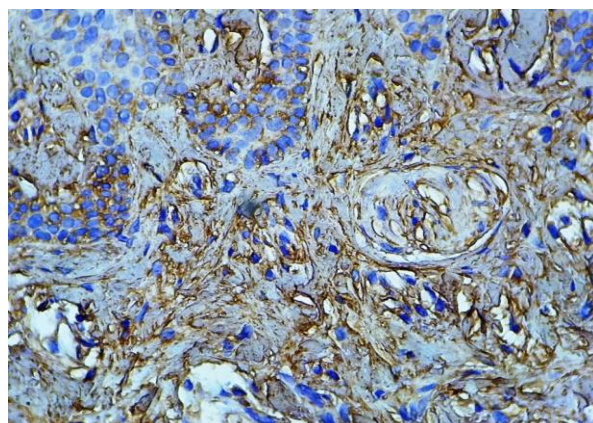


Fig. 3: Photomicrograph showing TLR 4 positivity in healthy group (40x)

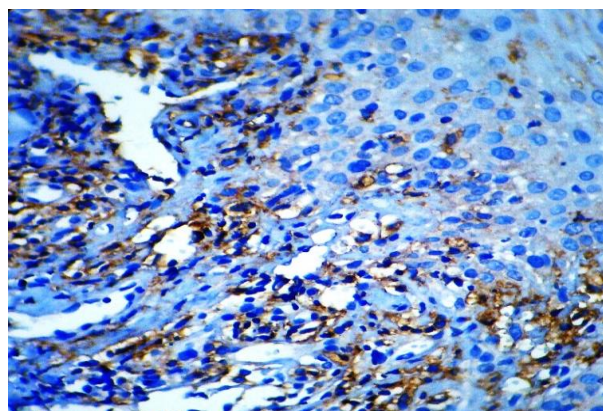


Fig. 2: Photomicrograph showing inflammatory cells positive for CD14 in chronic periodontitis group (40x)

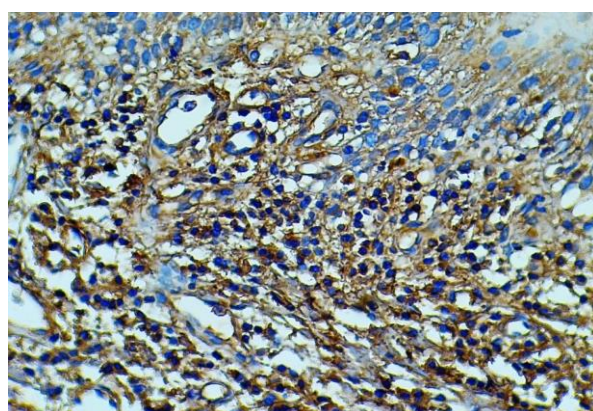


Fig. 4: Photomicrograph showing inflammatory cells positive for TLR 4 in chronic periodontitis group (40x)

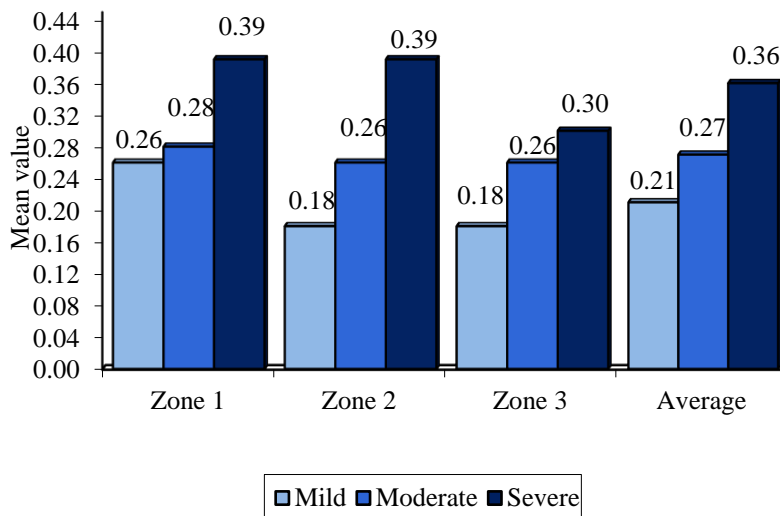


Fig. 5: Comparison of histological grading in chronic periodontitis groups with respect to TLR 4 scores at zone 1, zone 2, zone 3 and their average

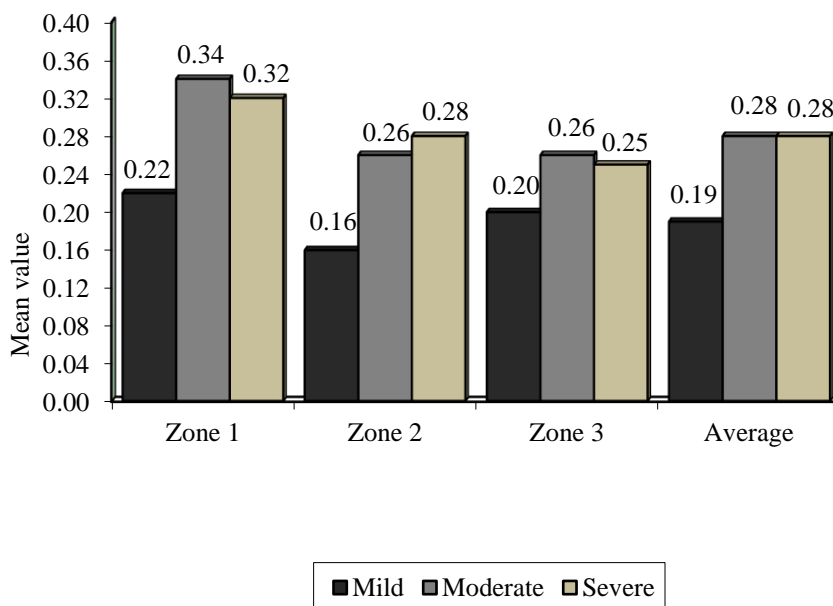


Fig. 6: Comparison of histological gradings in chronic periodontitis groups with respect to CD14 scores at zone 1, zone 2, zone 3 and their average

Discussion

Toll-like receptors are primarily expressed on cells of the innate immune system, which help them to

induce a large variety of immune responses to specific pathogens.⁽⁷⁾ Neutrophils is the major innate immune cells which express Toll-like receptor 1, Toll-like

receptor 2, and Toll-like receptor 4. Resembling neutrophils, macrophages/monocytes is also considered as a first line of defense against microbial pathogens.⁽⁸⁾

During inflammation, Toll-like receptors of resident immature dendrite cells detect the pathogen-associated molecular patterns of or released from invading microorganisms.⁽⁹⁾ Upon interaction information are transmitted through signaling pathways, resulting in activation of dendrite cells. This activation involves expression of co-stimulatory molecules and production of cytokines, all of which are critical of T-cell priming and differentiation.⁽⁹⁾ Innate immunity also faces the challenge of discriminating the host from among a large number of periodontal pathogens using a limited number of cell surface receptors.⁽⁹⁾

One complicating factor is that microorganisms have the ability to mutate in order to escape host recognition. Innate immunity meets this challenge to recognition of evolutionarily conserved structures – known as PAMPs – on pathogens that are not present in higher eukaryotes. These play a role in the ability of pathogens to evade host defense, and are therefore not subject to high mutation rates. These PAMPs are shared among pathogens but are not expressed by the host. Thus, a limited number of receptors is able to recognize a large number of pathogens.⁽¹⁰⁾ TLR2s and 4 along with CD14 as a co-receptor, are involved in Gram-positive and Gram negative PAMP recognition. Activation of these receptors there is transcription of various pro-inflammatory cytokines that have been associated with periodontitis.⁽¹¹⁾

We conducted a study to determine the level of expression of TLR4 and CD14 in healthy and chronic periodontitis and to correlate with the histological mild, moderate and severe inflammatory groups.

The results of the present study showed, using immune histochemical technique, that TLR 4 and CD14 is expressed in human gingival tissue. The results also confirmed and extended previous evidence^(12,13,14) showing the expression of TLRs in the various types of cells in healthy human gingival tissue. This expression is significantly exacerbated in patients with periodontal disease.

In the present study it was observed that the TLR 4 and CD14 was expressed both in healthy and chronic periodontitis group. TLR 4 and CD14 expression was significantly higher in chronic periodontitis group compared to healthy group.

Our data coincides with previous reports^(11,13,14) showing that the inflamed periodontium is infiltrated by TLR-expressing inflammatory cells, whereas healthy gingival tissue displays significantly lower levels of TLR expression. Likewise, our immunohistochemical data are in agreement with results stated by Hans et al and Beklen et al showing that gingival epithelial cells also express TLRs.^(12,15)

According to Sarah SM et al⁽¹⁹⁾ TLR4 was significantly increased in chronic periodontitis. This has

provided new insight into the mechanism of innate immunity to microbial pathogens. Commencement of TLRs leads to the induction of the antimicrobial pathways as well as the up regulation of antigens presentation molecules and secretion of cytokines that influence the nature of adaptive immune response. Chronic stimulation of TLRs in periodontal tissues by bacterial PAMPs can lead to excessive production of pro-inflammatory mediators, resulting in tissue destruction. Also, periodontitis induced by bacterial plaque may start with disruption and penetration of the gingival epithelial barrier by invasive bacteria or their cytotoxic products.⁽¹²⁾ Through this invasion into deeper tissues, TLRs in other cells such as macrophages, fibroblasts, osteoblasts, osteoclasts and antigen-presenting cells become activated. These cells, when stimulated, produce various pro-inflammatory cytokines that lead to inflammation and immune cell infiltration. The infiltrated cells, such as memory T-cells, further produce cytokines and amplify the inflammatory reaction, leading to destruction of connective tissue and bone.⁽¹⁷⁾

The results of our present study showed that expression of TLR4 and CD14 increased in zone 1 followed by zone 2 and zone 3. This is in agreement with the study of Becerik S et al⁽¹⁸⁾ also who showed that TLR 4 and CD14 was significantly higher in zone 1 (connective tissue subjacent to the pocket epithelium) compared to zone 2, in diseased periodontal tissues. This suggests that expression of TLR4 and CD14 are due to stimulation from a variety of bacterial plaque components and inflammatory cytokines in that zone.

Extensive evidence shows that LPS are an important etiologic factor in the pathogenesis of periodontitis. It has been reported that the local concentrations of LBP and CD 14 in gingival tissues may be the arbiter for the immunobiological effects of LPS in periodontal disease.⁽¹⁹⁾

The expression of CD14 may be reflection of periodontopathogens and/or cytokine interactions.⁽¹³⁾ This was also supported by an in vitro study performed by Palucka et al⁽²⁰⁾ stating that a conversion potential might exist among the CD14 positive cells in the presence of differentiation and stimulatory signals such as LPS and cytokines. It is well recognized that LPS constitute one of the major virulent factors of the surface of pathogenic gram negative bacteria and interact with host cells via pattern recognition receptors such as LBP, CD14 and TLRs.⁽²⁰⁾ CD14 mediates the production of cytokines such as tumor necrosis factor (TNF), IL-6 and IL-8 induced by LPS. This information implies the significance of the mechanism of CD14 in chronic periodontitis.

The results of our study indicate that TLR4 expression of healthy gingival tissues was lower than in the tissues of patients with periodontal disease. TLR4 was expressed strongly in the epithelium and

connective regions of gingival tissues from patients with periodontitis. This is in accordance with the study conducted by Botello R et al.⁽²¹⁾

The observation from the data collected in our study showed the expression of CD14 was detected in connective tissue underlying the pocket epithelium which is in relation to results of Lei Ren et al.⁽¹³⁾ This suggests that in zone 1, dendritic cells may undergo maturation and migration possibly towards the zone of active inflammation and destruction.⁽¹³⁾

The higher ratio of TLR 4 and CD 14 positive cells near the pocket epithelium i.e. zone 1 may have resulted from the stimulation from the variety of bacterial products and inflammatory cytokines.⁽¹⁸⁾

TLR4 are uttered to cells that respond to Lipopolysacchride, these cells on activation inflammatory cytokines through toll like receptors. Therefore, TLR4 plays an essential task in innate immune mechanisms. TLR signaling results from innate immune responses involving the release of the antibacterial β -defensins cathelicidin and calprotectin, as well as neutrophil chemoattractant (interleukin-8).⁽²²⁾

Ulevich et al suggested that TLR4 is the main protein involved in recognition of gram negative bacteria via interaction with lipopolysacchride.⁽²³⁾ TLR signaling confine and prevents commensal organisms, thereby maintaining gingival health.

In the current study the results indicate that positive correlation exists on the TLR 4 and CD 14, suggesting a potential interlinked with the two.

The chronic periodontitis group, in zone 1 there was a high correlation of TLR4 with all zones of CD14. Mori et al⁽¹⁴⁾ also described that TLR 4 was the highest in zone 1 relative to both other zones.⁽¹⁴⁾ Hamada et al⁽²⁴⁾ stated that TLR 4 was significantly higher expressed in connective tissue adjacent to the pocket epithelium. In this layer the structure of subgingival plaque consisted of dense layers of gram positive organisms, spirochetes and other motile forms at the base of the pocket. Our results are comparable to those of Hamada et al as we also found that TLR 4 was expressed most in zone 1.⁽²⁴⁾

The observation in zone 2 of our study specimens showed no significant correlation between TLR4 and CD14 in chronic periodontitis group.

The observation made in our study showed that TLR 4 expression in zone 3 and CD14 expression in zone 2 and 3 were significantly correlated. This could be due to the fact that zone 3 is adjacent to the oral epithelium and is affected by bacterial plaque and antigenic stimulants.⁽²¹⁾

On examining data of the healthy group of our study, it was observed that TLR4 and CD14 were expressed in healthy group as well, although correlation was insignificant. This could be explained as the expression of TLR4 and depends on the extent of the inflammatory infiltrate.^(21,22,25) It has been suggested recently that the oral mucosa develops tolerance after

repeated exposure to bacterial products. Down regulation of TLR expression and inhibition of intracellular signaling may be the underlying mechanisms of acceptance.⁽²²⁾

Kusumoto Y et al observed that TLR 4 was primarily detected in connective tissue. In healthy gingival tissue only weak expression was detected.⁽²⁶⁾ Overall these observations are consistent with our findings that TLR4 expression is related to the severity of periodontal inflammation. Our results are in accordance with the study done by Becerik S et al,⁽¹⁸⁾ Mori Y et al,⁽¹⁴⁾ Ren L et al⁽¹³⁾ where TLR 4 and CD14 expression was higher in chronic periodontitis compared to healthy.

In contrast Mori et al⁽¹⁴⁾ showed that CD14 expression was confined to the cells around the epithelium connective interface and its expression levels in periodontal tissue was significantly lower than in healthy control tissue. Our results on the other hand indicated that CD14 expression increased in disease than in health.

In the current study when the TLR 4 and CD 14 immunohistochemical localization was compared with the histologic gradings the highest positivity was observed for TLR4 in severe inflammatory group. The CD14 expression was higher in severe and moderate inflammatory group of chronic periodontitis. As there is increased from the inflammatory infiltrate mainly due to the bacterial plaque component and antigenic stimulus, the positivity of TLR 4 and CD 14 expression increases.⁽²³⁾ In the present study CD14 increased positivity was up regulated in moderate and severe inflammatory group. In contrast Mori et al⁽¹⁴⁾ demonstrated that CD14 positive cell ratio was lowest in zone 1 of severe group. He suggested that CD14 is down regulated in pathogenesis of periodontitis and/or that dendritic cells undergo maturation and migration in periodontitis.

Periodontal health represents a dynamic state in which pro-inflammatory and anti-microbial activities for control of infection are optimally balanced by anti-inflammatory mechanisms to prevent unwarranted inflammation. This homeostasis is disrupted when pathogens present in dental plaque undermines the host defense mechanism.^(23,24) Chronic stimulation of TLR4 and CD14 in periodontal tissues by bacterial PAMPs can lead to excessive production of pro-inflammatory mediators, resulting in tissue destruction.⁽²⁴⁾

The results of our study indicate that the level of TLR4 and CD14 showed increased expression in chronic periodontitis. However, further studies are needed to elucidate the precise role of TLRs in the pathogenesis of periodontal disease.

Also, periodontitis induced by bacterial plaque may start with disruption and penetration of the gingival epithelial barrier by invasive bacteria or their cytotoxic products. Through this invasion into deeper tissues, TLR 2 and 4 along with the CD14in other cells such as

macrophages, fibroblasts, osteoblasts, osteoclasts and antigen-presenting cells become activated.⁽²⁴⁾ Stimulated cells produce various pro-inflammatory cytokines that lead to inflammation and immune cell infiltration. The reaction is further amplified by memory T cells leading to destruction of connective tissue and bone.⁽²⁴⁾ This probably indicates that, TLRs act as a two-edged sword, not only maintaining periodontal health but also contributing to periodontal tissue destruction.

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