# Expression of inflammatory mediators in CD103+ and CD103– T cells isolated from periodontitis tissues: a pilot study

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## Abstract

**Introduction**: Despite the important role of tissue - resident memory T ($T_{RM}$) cells in immunity and pathology of mucosal tissues, the study of tissue-resident memory T cells in periodontitis tissues has been limited.

**Objectives**: We investigated the presence of $T_{RM}$ cells in periodontitis by staining their surface marker; CD103 and then analyzed by flow cytometry. The production of IFN-γ and IL-17 by CD103+ and CD103- T was also investigated.

Materials and Methods: Human periodontal tissues were obtained from patients with severe chronic periodontitis. Expression of CD103, IFN-γ and IL-17 was analyzed by 6-color flow cytometry.

**Results**: Majority of infiltrated T cells in periodontitis tissues were CD4+ T cells. Expression of CD103 was mainly detected on CD8+ T cells. CD103+ and CD103- CD4+ T cells produced IFN-γ and IL- 17 whereas CD103+ and CD103- CD8+ T cells produced only IFN-γ.

**Conclusions**: Expression of CD103 was detected on CD8+ T cells and only minimal on CD4+ T cells. The ability of CD103+ and CD103- T cells to produce IFN-γ and IL-17 suggests their possible role in periodontal inflammation and bone resorption.

***Keywords***: *CD103, inflammatory mediators, periodontitis, T cells, tissue - resident memory T cells*

## Introduction

Periodontitis is a common chronic inflammatory disease of tooth supporting structure which includes gingiva, cementum, periodontal ligament and alveolar bone. The disease pathogenesis involves host immune response to bacterial dental plaque. Abundant of adaptive immune cells such as T and B cells, are found in periodontal lesions. Seymour *et al.* (1979)[1] described a clinical transition from gingivitis (a mild/moderate form of periodontal disease) to periodontitis (a severe form) is associated with a change from a T cell-dominated lesion to B cell and plasma cell- dominated lesion. High levels of pro-inflammatory mediators and cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-1β, IL-17, receptor for activator of nuclear factor kappa-B ligand (RANKL) have been observed in periodontitis lesion.[2]

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It is now recognized that T cells population are heterogeneous. T cell subsets include Th (T helper)1, Th2, Th17, and Treg (regulatory T cells). Th1 cells mediate predominantly cell-mediated immune response to intracellular pathogens by secreting IFN-γ, IL-2 and TNF-γ. Conversely, Th2 cells have a role in growth and differentiation of activated B cells by secreting IL-4, IL-5, IL-10 and IL-13.[3] Th1 cells were hypothesized to associate with stable gingivitis lesion, while Th2 cells were associated with progressive periodontitis lesion.[4] However, some studies showed predominantly Th1 response over Th2 in diseased periodontal tissue[5] and Th1 role in periodontal bone resorption.[6] At present, the role of Th1 and Th2 cells in periodontal disease remains in conflict. More recently, other Th subsets, including Th17 cells and regulatory T cells (Treg) have been observed in periodontitis tissues. Th17 secretes IL-17 which promotes periodontal inflammation and destruction.[7, 8] Whereas Treg secretes IL-10 and TGF-β and oppositely against Th17 cells by inhibiting inflammation and self-tolerance.[9]

Following positive and negative selection, T cells are released from the thymus as mature naïve T cells harboring a given epitope specificity. In response to cognate antigen encounter, naïve T cells proliferate and differentiate into effector cells, the vast majority of which migrate to peripheral tissues and inflamed sites to facilitate destruction of infected targets.[10] Following antigen stimulation, more than 95% of the effector cells die while a small pool of T cells ultimately develops into long-lived memory T cells.[11] The memory T cells found in blood can be divided into subsets based on the differential expression of markers of migration, CD62L and CCR7.[10] In recent years, a new subset of memory T cells that permanently reside in non-lymphoid tissues has been identified; they are now widely referred to as tissue-resident memory T ($T_{RM}$) cells.[12-14] The mechanism of $T_{RM}$ cell retention in tissues is not precisely known. $T_{RM}$ cells in mice, nonhuman primates and humans express CD103 and CD69. The ligand for CD103, E-cadherin, is expressed on epithelial cells suggesting that the interaction of CD103 and E-cadherin may contribute to maintaining the resident status of $T_{RM}$ in peripheral tissue.[15] $T_{RM}$ provides superior protection against viral infection relative to the circulating memory T cell.[16-18] Evidences showed that $T_{RM}$ play roles in protection from infection in skin,[16] lungs,[19] GI tracts[20] and vagina.[21] $T_{RM}$ cells can respond rapidly to pathogen challenge at infected sites independently of recruitment of T cells from the blood.[18] However, these cells have also been reported in causing diseases such as psoriasis and fixed drug eruption.[22, 23] The immunopathology of psoriasis is thought to be due to significant expression of IL-17 and IL-22 by $T_{RM}$ cells[22] whilst fixed drug eruption involves in high expression of IFN-γ by $T_{RM}$.[23]

So far, there have been very limited studies regarding the role of memory T cells in periodontal disease. Early studies demonstrated that majority of T cells in periodon-titis tissue expresses memory cell phenotype. Preliminary flow cytometric analysis from our laboratory revealed the presence of different memory T cell subsets including stem cell memory T cells ($T_{SCM}$), central memory T cells ($T_{CM}$), effector memory T cells ($T_{EM}$), terminal effector memory T cells ($T_{TE}$), and $T_{RM}$ in periodontal tissue, both in health and disease. Immunohistochemical analysis showed that CD103+ $T_{RM}$ cells were localized in both epithelial layer and connective tissue. $CD8^+$T cells were mainly detected in epithelial layer, while $CD4^+$ cells were mainly detected in connective tissue. Further investigations are needed in order to understand the function of periodontal tissue-resident memory T cells in periodontitis in terms of their expression of pro-inflammatory mediators/cytokines which involves in tissue inflammation and bone destruction.

## Objectives

The aim of this study was to analyze the expression of pro-inflammatory mediators (IFN-γ and IL-17) by $T_{RM}$ cells isolated from periodontitis tissues using flow cytometry.

## Materials and methods

### Reagents

Roswell Park Memorial Institute (RPMI)-1640 and Dulbecco's phosphate-buffered saline (DPBS) was obtained from Gibco (Grand Island, NY, USA). Fetal calf serum, collagenase, phosphate-buffered saline (PBS) and Staphylococcal enterotoxin B (SEB) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### Monoclonal antibodies

Fluorescence-conjugated mouse anti-human CD3, anti-human CD4, anti-human CD8, anti-human CD103, anti-IFN-γ and anti-IL-17 monoclonal antibodies and mouse isotype control monoclonal antibodies were obtained from BD sciences (San Jose, CA, USA).

### Subjects selection, periodontal tissue collection and ethical consideration

Periodontitis tissue specimens were collected from patients at Periodontal Clinic and Department of Oral Maxillofacial surgery, Faculty of Dentistry, Chulalongkorn University. The ethical approval by the ethics committee of Faculty of Dentistry, Chulalongkorn University and informed consent of all participating subjects was obtained before the operation (ethical approval number 119/2016). Periodontal tissue samples were collected from periodontitis subjects at the Periodontal Clinic or Oral Surgery Clinic, Faculty of Dentistry, Chulalongkorn University. Gingival tissues surrounding teeth with other dental diseases such as pulpal diseases were excluded. All subjects were in good general health and none of them had taken antimicrobial or anti-inflammatory drugs within the previous 3 months. Each subject had no history of periodontal treatment in the past 6 months.

Periodontitis tissues were obtained from a site of extracted teeth with hopeless periodontal prognosis[24] (gingival inflammation, clinical attachment loss 5 millimeters or more and bone loss 50% of the root length or more).

The excised periodontal tissue specimen (size 3 mm3) was immediately placed in a sterile tube that contains RPMI-1640 medium and then transferred to the laboratory within a few hours for further study.

### Gingival cell preparation and flow cytometric analysis

Tissues were washed thoroughly and cut into small fragments (1 - 2 $mm^3$). Then, they were incubated in 2 mg / ml of collagenase (Sigma Chemical Co.) for 90 minutes at 37°C. Residual tissue fragments were disaggregated by flushing several times with pipette to obtain single cell suspensions and filtered through filter of mesh size 70 µm and 40 µm (Becton Dickinson).

Expression of cytokines in T cells isolated from periodontitis tissues was determined. Intracellular cytokine staining (ICS) including IFN-γ and IL-17 was assessed on $CD103^+$ and $CD103^-$ T cells. Gingival cell suspensions from periodontal tissue specimens were stimulated with SEB, a superantigen that triggers polyclonal T cell activation and massive cytokine release and sample with no stimulation served as negative controls. The cells were stained with cell surface markers, washed and treated with fixation/permeabilization solution (BD Pharmingen) and then stained with mAbs against cytokines. The stained cells were analyzed by flow cytometry (BD FACSCelestaTM, Becton Dickinson).

### Statistical Analysis

This was a pilot study and did not require statistical analysis. Data were analyzed and were presented as mean ± SE.

## Results

Determination of CD3, CD4, CD8 and CD103 positive T cells in periodontitis tissues by flow cytometry

Flow cytometric analysis of infiltrated immune cells in periodontitis tissues (Fig. 1A) demonstrates that about 44% of lymphocytes were CD3+ T cells. Majority of T cells were CD4+ T cells (53.83%), while 30% of them were CD8+ T cells (Fig. 1B). The percentage of CD4+ T cells that expressed tissue-resident marker, CD103 was low (3.7%). However, greater percentage of cells expressing CD103 was detected in CD8+ T cell population (27%).

### Cytokine production of $CD103^+$ and $CD103^-$ T cells in periodontitis

To investigate the cytokine profiles of $CD103^+$ and CD103- T cells isolated from periodontitis, we assessed the expression of IFN-γ and IL-17 using intracellular cytokine staining following polyclonal stimulation with SEB. Flow cytometry analysis in Fig. 2 shows no expression of IFN-γ and IL-17 in control unstimulated samples, while samples stimulated with SEB were able to produce both cytokines. $CD103^+$ and $CD103^-$ $CD4^+$T cells produced either IFN-γ alone or IL-17 alone, whereas the production of IFN-γ plus IL-17 was negligible. In contrast, $CD103^+$ and CD103- $CD8^+$T cells produced only IFN-γ, but not IL-17. Greater proportion of $CD4^+$ and $CD8^+$T cells expressing CD103 produced more IFN-γ (1.7 -3.7 fold) compared to those that had no CD103 expression.

## Discussion

Large infiltration of lymphocytes is commonly observed in periodontitis tissues. We detected more numbers of $CD4^+$ T cells (54%) compared to $CD8^+$ T cells (30%). These findings are consistent with results from previous reports.[25] The role of local tissue immunity has received more attention lately primarily due to the discovery of a new subset of memory T cells termed tissue-resident memory ($T_{RM}$) cells. These long-lived and non-recirculating $T_{RM}$ cells permanently reside in

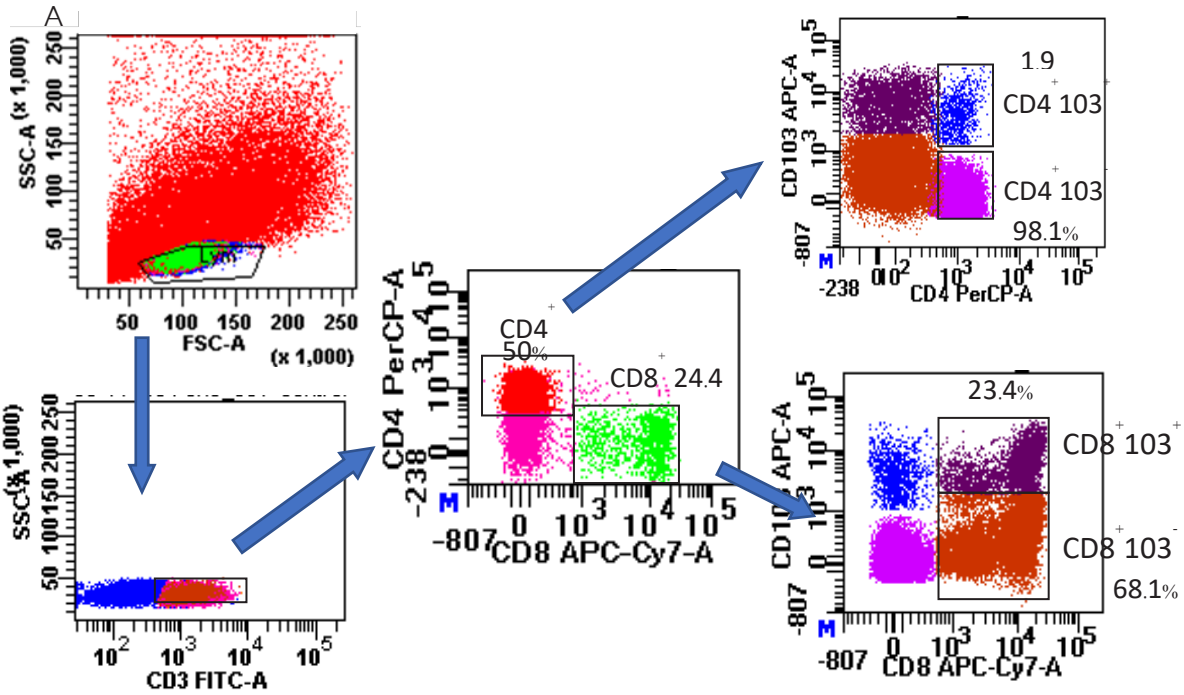


Fig. 1A Expression of CD103 on T cells. Flow cytometry gating strategy to identify CD103 expressing T cells isolated from periodontitis tissues.

B

| | $CD103^+$ Mean±SE* | $CD103^-$ Mean±SE* |
|---|---|---|
| CD4+ (53.83±4.06) | 3.70±1.12 | 79.06±15.34 |
| CD8+ (29.99±3.11) | 26.95±8.77 | 60.71±9.77 |

*Standard error

Fig.1B Mean percentage of CD103+ and CD103- in CD4+ and CD8+ T cells (n=3).

non-lymphoid tissues including skin, brain, vagina and lung, and provide rapid, effective local protection against reinfection relative to circulating counterpart memory T cells[18, 21, 26-28]. This novel memory T cell subset express CD103 ($\alpha E\beta 7$ integrin) and CD69 (C-type lectin), both of which are involved in cell adhesion and tissue retention.[29]

There has been very little study of T cells expressing CD103 in periodontitis. The presence of $CD103^+$ T cells in periodontitis tissues was first described by immunohistochemical staining more than 20 years ago.[30] In our study, we found greater number of $CD8^+$ T cells in periodontitis tissues expressed CD103 compared to $CD4^+$ T cells. These results are in line with other observations suggesting that $CD4^+$ T cells poorly express CD10331

The production of cytokines; IFN-γ and IL-17 from $CD103^+$ and $CD103^-$ T cells in periodontitis has never been studied. Expression of IFN-γ has been consistently reported in periodontitis tissues and may involve in tissue inflammation by recruitment of circulating memory T and B cells via VCAM-1 pathway[31]. IL-17 has been proposed as a major driving force of bone in periodontitis through the upregulation of RANKL and the activation of osteoclastogenesis.[32] Here we show that both $CD103^+$ and CD103- $CD4^+$ T cells produced more IFN-γ than IL-17. Interestingly, $CD103^+$ and CD103- $CD8^+$ T cells produced only IFN-γ, but not IL-17. The ability to produce IL-17 seems to restrict to $CD4^+$ T cells in periodontal tissues.[33] However, it has been recently demonstrated

A

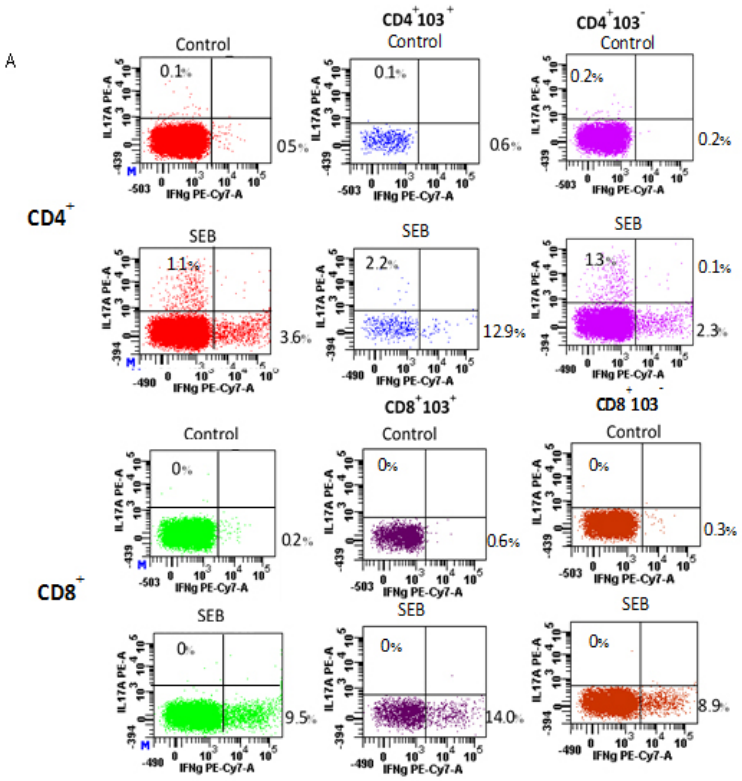


Fig.2A Flow cytometry analysis of IFN-γ and IL-17 produced by CD103$^+$ and CD103- CD4$^+$ and CD8$^+$ T cells.

B

| | IL-17 Mean±SE* | | IFN-γ Mean±SE* | |
|---|---|---|---|---|
| | 103$^+$ | 103$^-$ | 103$^+$ | 103$^-$ |
| CD4$^+$ | 1.34±0.55 | 1.34±0.65 | 14.75±9.65 | 3.93±2.60 |
| CD8$^+$ | 0.07±0.04 | 0.03±0.02 | 11.32±4.46 | 6.66±2.78 |

*Standard error*

Fig. 2B Mean percentage of IFN-γ, and IL-17 produced in each subset of T cells (n=3).

that CD103$^+$ CD8$^+$ T cells from skin lesions of patients with psoriasis were able to produce IL-17.[34] It is still unclear why CD8$^+$ T cells from periodontitis tissues and psoriasis lesions are different regarding to IL-17 production. Local tissue environment and stimulating antigen (s) could influence the cytokine profiles.

## Conclusion

In conclusion, we observed infiltration of CD4$^+$ and CD8$^+$T cells in periodontitis tissues. CD103 expression was observed more on CD8$^+$T cells than CD4$^+$T cells. CD103$^+$ and CD103- CD4$^+$T cells were able to produce IFN-γ, and IL-17, whereas CD103$^+$ and CD103$^-$ CD8$^+$T cells produced only IFN-γ. The ability to produce such cytokines suggests their possible role in periodontal inflammation and bone destruction observed in periodontitis. Further study is required to better understand the role of non-recirculating tissue-resident memory T cells and recirculating memory T cells in immunopathology of periodontitis.

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