รายงานวิจัยฉบับสมบูรณ์

โครงการ การวิเคราะห์ที่ครอบคลุมถึงชนิดกลุ่มย่อยของเมม โมรี ที เซลล์ในเนื้อเยื่อปริทันต์ปกติและที่เป็นโรคปริทันต์

โดย รังสินี มหานนท์ และคณะ

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและจุฬาลงกรณ์มหาวิทยาลัย

Abstract

Human gingiva is a primary site that constantly challenged by dental plaque microbiome, therefore effective and balanced innate and adaptive immune responses are crucial to maintain periodontal tissue homeostasis.We investigated heterogeneity of human memory T cells in healthy gingiva and periodontitis. Immunostaining of healthy gingiva showed large clusters of $CD3^+$ T cells in the connective tissue adjacent to junctional epithelium. Flow cytometric analysis showed that more than 95% of gingival $CD3^+$ T cells expressed memory cell phenotype both in health and periodontitis. Healthy gingiva- memory T cells are consisted of two populations, including $CD69^-$ recirculating and $CD69^+$ gingiva-resident memory T cells. In both populations, $CD4^+$ T cells with transitional memory phenotype ($CD28^+$, $CD95^+$, $CCR7^-$ and $CD45RA^-$) constitute the major subset. In periodontitis tissues, larger aggregates of CD3+ T cells were detected at the base of periodontal pocket, especially apically toward the advancing front of the lesion. A significant increase in the proportion of $CD4^+CD69^+CD103^-$ memory T cells was observed in periodontitis tissue as compared to control healthy gingiva. $CD4^+$ memory T cells from periodontitis tissues produced either IL-17 alone or IFN-γ alone, whereas $CD8^+$ memory T cells produced only IFN-γ. Our findings suggest that recirculating and gingiva-resident memory T cells could represent an important part of immune surveillance network in the connective tissue of healthy gingiva. A dysbiosis in subgingival plaque bacteria could disrupt or damage gingival barrier leading to uncontrolled T cell activation and cause deleterious tissue inflammation and bone loss. More studies are needed to better understand how subgingival microbiota and gingival-specific signals effectively regulate immune responses in periodontal health and disease.

บทคัดย่อ

เหงือกของมนุษย์เป็นบริเวณแรกที่ถูกท้าทายตลอดเวลาด้วยไมโครไบโอมของคราบจุลินทรีย์ ด้วยเหตุนี้ประสิทธิภาพและสมดุลของการตอบสนองของระบบภูมิคุ้มกันเป็นสิ่งสำคัญในการรักษาสภาวะสมดุลของเนื้อเยื่อปริทันต์ คณะผู้วิจัยทำการศึกษาถึงความหลากหลายของเมมโมรี ที เซลล์ในเหงือกที่มีสภาวะปริทันต์ปกติและที่เป็นโรคปริทันต์ การย้อมทางอิมมูโนของเหงือกที่มีสภาวะปริทันต์ปกติแสดงให้เห็นกลุ่มใหญ่ของเซลล์ ซีดี 3+ ที เซลล์ในเนื้อเยื่อเกี่ยวพันที่อยู่ติดกับเยื่อบุผิวเชื่อม การวิเคราะห์ด้วยวิธีโฟลไซโทเมทรีแสดงว่ามากกว่า 95 เปอร์เซ็นต์ของซีดี 3 ทีเซลล์จากเหงือกแสดงฟีโนไทป์เป็นเมมโมรีเซลล์ทั้งในสภาวะปริทันต์ปกติและที่เป็นโรคปริทันต์ เมมโมรี ที เซลล์ในเหงือกประกอบด้วย 2 กลุ่ม คือ ซีดี 69^- รีเซอคิวเลทติ้งเมมโมรี ที เซลล์และ ซีดี 69^+ เรสซิเด็นซ์เมมโมรี ที เซลล์ โดยทั้งสองกลุ่ม ซีดี 4^+ ทีเซลล์ที่มีฟีโนไทป์แบบทแรนซิชันแนลเมมโมรี ที เซลล์ (ซีดี 28^+ ซีดี 95^+ ซีซีอาร์ 7^- ซีดี 45 อาร์เอ$^-$) เป็นกลุ่มย่อยที่มีมากที่สุด ในเนื้อเยื่อเหงือกที่เป็นโรคปริทันต์พบว่า ซีดี 3+ ที เซลล์รวมกลุ่มขนาดใหญ่บริเวณส่วนฐานของร่องลึกปริทันต์ โดยเฉพาะอย่างยิ่งส่วนยอดใกล้แนวของรอยโรค พบการเพิ่มขึ้นอย่างมีนัยสำคัญของสัดส่วนของซีดี 4^+ ซีดี 69^+ ซีดี 103^- เมมโมรี ที เซลล์ในเนื้อเยื่อเหงือกเป็นโรคปริทันต์เมื่อเปรียบเทียบกับเนื้อเยื่อเหงือกที่สภาวะปกติ ซีดี 4^+ เมมโมรี ที เซลล์จากเนื้อเยื่อเหงือกเป็นโรคปริทันต์ผลิต IL-17 หรือ IFN-γ เพียงอย่างใดอย่างหนึ่ง ในขณะที่ซีดี 8^+ เมมโมรี ที เซลล์ผลิตเพียง IFN-γ เท่านั้น ผลการวิจัยของคณะผู้วิจัยชี้ให้เห็นว่าทั้งรีเซอคิวเลทติ้และเรสซิเด็นซ์เมมโมรี ที เซลล์เป็นส่วนสำคัญของเครือข่ายการเฝ้าระวังด้านภูมิคุ้มกันในเนื้อเยื่อเกี่ยวพันของเหงือกสภาวะปกติ สภาวะขาดความสมดุลของคราบจุลินทรีย์ใต้เหงือกสามารถทำให้ระบบป้องกันของเหงือกเสียหรือทำลายนำไปสู่การกระตุ้นที เซลล์จนไม่สามารถควบคุมได้และเป็นสาเหตุของอักเสบของเนื้อเยื่อและสูญเสียกระดูกที่ร้ายแรง ยังต้องการการศึกษาอีกมากเพื่อความเข้าใจว่าจุลินทรีย์ที่อาศัยอยู่ใต้เหงือกและสัญญาณเฉพาะในเหงือกมีประสิทธิภาพในการควบคุมการตอบสนองของระบบภูมิคุ้มกันในเนื้อเยื่อปริทันต์ปกติและที่เป็นโรคปริทันต์อย่างไร

หน้าสรุปโครงการ (Executive Summary)

ชื่อโครงการ การวิเคราะห์ที่ครอบคลุมถึงชนิดกลุ่มย่อยของเมมโมรี ที เซลล์ในเนื้อเยื่อปริทันต์ปกติและที่เป็นโรคปริทันต์

Comprehensive analysis of periodontal tissue memory T cell subset in health and disease

Rational

Periodontal disease is a common chronic inflammatory disease in humans, which affects gingiva and supporting bone. It is recognized as one of the major oral health problems and may lead to tooth loss in a severe case (periodontitis). The disease results from host immune response to bacterial biofilms in dental plaque. The abundant of lymphocyte infiltrates has been detected in periodontal lesions. A mild/stable lesion (gingivitis) is dominated by T cells while periodontitis is dominated by B cells. Shifting from a T cell-gingivitis lesion to a B cell-periodontitis lesion is hypothesized to be associated with pathogenesis of periodontal disease and this may be due to the presence of specific T cell subsets and their response to periodontal pathogens. Recent advance in memory T cells has revealed that these cells constitute the most abundant lymphocyte population in the human body such as blood, lymphoid and non-lymphoid tissues. Memory T cells has subset heterogeneity. While circulating memory T cells (central memory T cells, effector memory T cells, and terminal effector memory T cells) provide efficient protection against systemic infections, their ability to deal with localized infections in the periphery is often limited. A newly identified memory T cells that permanently reside in non-lymphoid tissues such as mucosal sites (skin, lung, gut, and vagina) are referred to as tissue-resident memory T cells. Recently it has been highlighted that these tissue-resident memory T cells are capable to provide superior protection against infection relative to the circulating memory T cells. So far, there has been no study of periodontal tissue memory T cell subsets. We therefore, propose to comprehensively investigate the memory T cell subsets reside in healthy and diseased periodontal tissue. We will also evaluate each subset upon their cytokine response and expression of granzyme B, all of which involve periodontal tissue inflammation and bone destruction. Such a study will provide further insights into periodontal tissue memory T cells and their role in disease.

วัตถุประสงค์

We will investigate T cells isolated from periodontitis tissue and compare with healthy periodontal tissue in order to provide new insights into subsets of memory T cells in human periodontitis and their specific cytokine response. The specific objectives are as follows:

1. Flow cytometric analysis of memory T cell subsets (T_{SCM}, T_{CM}, T_{EM}, T_{TE}, T_{RM}) in periodontitis tissue as compared with healthy periodontal tissue.
2. To investigate the expression profile of cytokines (IL-2, TNF-α, IFN-γ, RANKL), granzyme B in different subsets of periodontal tissue memory T cells.
3. To study anatomical localization of periodontal tissue T_{RM} that express CD103 by immunohistochemistry

สรุปผลการดำเนินงาน

1. We evaluated the memory subsets of T cells from both healthy periodontal tissue and periodontitis specimens. We found T_N, T_{SCM}, T_{CM}, T_{EM}, T_{TE}, T_{RM} cells in both oral healthy and periodontitis tissues.
2. We investigated the localization of CD3, CD4, CD8 (T cells markers) and CD103 (Resident T cells marker) in oral healthy and periodontitis section by immunohistochemical staining technique. We found that the CD3, CD4, CD8 and CD103 was clearly observed in the epithelial layer and lamina propria of oral healthy and periodontal tissue.
3. We investigated the expression profile of cytokine (IFN-γ, IL-17) and granzyme B in different subsets of periodontal tissue memory T cells. We found $CD4^+CD103^+$ and $CD4^+CD103^-$ memory T cells isolated from periodontitis tissues produced either IL-17 or IFN-γ while $CD8^+CD103^+$ and $CD8^+CD103^-$ memory T cells produced only IFN-γ

Introduction to the research problem and its significance

Periodontal disease is one of the most common chronic inflammatory diseases in humans. The disease involves tooth supporting structures, gingiva and alveolar bone. The severe form of the disease called periodontitis is known as one of the major causes of tooth loss in adults. At present, the imbalance in host immune response to periodontal pathogens in dental plaque is considered as a key for the periodontal disease initiation and progression. The abundant of lymphocyte infiltrates has been detected in periodontal lesions. Gingivitis lesions (a mild and stable form of periodontal disease) are dominated by T cells while periodontitis lesions are dominated by B cells and plasma cells. Therefore, the shifting from a T cell-gingivitis lesion to a B cell-periodontitis lesion has been suggested to be associated with pathogenesis of periodontal disease and this may be due to the presence of specific T cell subsets and their response to periodontal pathogens.

It is well recognized that T cells play important roles in immune responses and function by directly secreting soluble mediators or cytokines. Early T cell studies revealed the existence of different T cell subsets including Th (T helper)1, Th2, Th17, and Treg (regulatory T cells) in periodontal lesions. However, the significance of these cell types in periodontal health and disease remain unclear and inconclusive. The concept of Th1 and Th2 was derived from mouse model and rarely found to be related to human diseases. Many studies of human T cells have been conducted on sample peripheral blood, not periodontal tissue where the immune response takes place. The field of T cell research in periodontal disease has been slow down for the past twenty years.

Until very recently, the improved and more sensitive immunological methods have provided update information about antigen specific memory T cells. Different memory T cell subsets have been characterized based on the differential expression of surface markers and cytokines. The memory T cells found in the blood can be divided into central memory (T_{CM}) T cells, effector memory (T_{EM}) T cells, and stem cell memory T (T_{SCM}) cells which retain stem cell-like properties. Another subset of memory T cells that permanently reside in non-lymphoid tissues has recently been identified; they are now widely referred to as tissue-resident memory T (T_{RM}) cells. The mechanism of T_{RM} retention in tissues is not precisely known. Unlike peripheral blood memory T cell subsets, T_{RM} represent the predominant memory T cell subset in mucosal sites (skin, gut, lung, and vagina), spleen and bone marrow. These T_{RM} cells are crucial as they provide a first line of defense against secondary infection by the same pathogen at the local sites. For example, infection with influenza virus leads to the generation of both resident and transient circulating memory T cells in the lungs. However, lung T_{RM} CD4 T cells and CD8 T cells show optimal protection against influenza

challenge compared with circulating memory T cells. Collective evidence from skin and vagina studies also suggests that T_{RM} in peripheral tissues play a key role in mediating T cell–dependent protective immunity against microbial pathogens. Furthermore, these findings also suggest that peripheral blood immune response may differ from those at the tissue sites where they are needed.

Despite recent advance in memory T cells in mucosal tissues, there has been no study of periodontal tissue-specific memory T cell subset. We therefore, propose to comprehensively investigate the memory T cell subsets reside in healthy and diseased periodontal tissue. We will also evaluate cytokine response and expression of granzyme B on different subset of memory T cells. Such a study will provide further insights into periodontal tissue-specific memory T cells and their role in disease. The Immunology laboratory, Department of Periodontology, Faculty of Dentistry, Chulalongkorn University has many unique characteristics, which enable to carry out this important study: 1) our laboratory has continued working with periodontal tissue immune response with measurable outputs, 2) our team has multi-year experience in obtaining human periodontal tissues and isolation of immune cells from those tissues, and 3) multi-color flow cytometry has already exist in the lab.

Our research team has recently carried out a pilot study to investigate memory T cell subsets in two periodontal tissue specimens, one from healthy periodontal subject and the other from periodontitis patient. Flow cytometric analysis in Figure 1 shows that different memory T cell subsets could be identified in both healthy and diseased tissues. Increased percentage of memory $CD4^+$ and $CD8^+$ T cells expressed CD103 (known as tissue retention marker) was found in periodontitis tissue as compared to healthy tissue.

Literature review

Periodontal disease is generally described as specific diseases that involve a tooth supporting structure. The periodontal diseases are infectious and recognized as one of the most common chronic inflammatory diseases in humans (Holmstrup et al., 2010; Williams, 1990). Formerly, periodontal diseases were simply categorized into two distinct forms based on disease severity, gingivitis and periodontitis. Gingivitis is inflammation of the gingiva (Lyons et al., 1950). Periodontitis is inflammation of the periodontium that extends beyond the gingiva and produces destruction of the connective tissue attachment of the teeth (Ranney, 1993).

Dental plaque biofilms have been well recognized as etiologic agents. The disease initiation and progression results from host response to plaque bacteria. In healthy periodontal tissue, low amounts of Gram-positive aerobes and facultative anaerobes, such as *Streptococcus* species and *Actinomyces* species, are found supragingivally (Moore and Moore, 1994). More accumulation of plaque leads to gingival inflammation (or gingivitis) with increased cellular infiltrates. T cells are the dominant cell type in gingivitis lesions. In contrast, in the more advanced form of periodontal disease, periodontitis, cellular infiltrates including numerous T and B cells are observed together with high levels of inflammatory mediators such as IL-1β, tumor necrosis factor-α (TNF-α), prostaglanding E2 (PGE2), and interferon-γ (IFN-γ) receptor activator of NF-κB ligand (RANKL), and granzyme B in tissues and gingival crevicular fluid (Belibasakis and Bostanci, 2012; Page et al., 1997; Seguier et al., 1999). These mediators have been known to be involved in tissue and bone destruction (Graves et al., 2011). B cells and plasma cells are the dominant cell type in periodontitis lesions, and numerous Gram-negative anaerobes are found in subgingival biofilms (Seymour, 1991). The differences in microbial compositions and quantities between health/gingivitis and periodontitis may influence the local inflammatory response. Key periodontal pathogens, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia*, which are frequently detected in deep periodontal pockets, are well recognized for their virulence as etiologic agents in human periodontitis (1996).

T cells in periodontal disease

Generally, the T cell population is divided into two main subsets, $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells or helper T (Th) cells have received much attention not only regarding their role in effective immune defense, but also regarding their participation in tissue inflammatory responses, through the cytokines they produce. About three decades ago, Mosmann et al. (Mosmann et al., 1986) first described distinct functional subsets of Th cells in mice: Th1 and Th2 based on their cytokine profiles. Th1 cells secrete IFN-γ and IL-2 and mediate predominantly cell mediated immune responses. In contrast, Th2 cells mediate humoral immunity by secreting IL-4, IL-5 and IL-13. An elegant experiment conducted in a mouse model demonstrated that mice resistant to infection by the intracellular parasite *Leishmania major* developed Th1 response whereas those susceptible to the infection develop Th2 response (Mosmann and Coffman, 1989). The simplicity of this Th1/Th2 paradigm of resistance/susceptibility in a mouse model then became attractive and encouraged further investigation into many human diseases including human periodontitis. It was hypothesized by Seymour and co-workers (Gemmell et al., 2002; Gemmell et al., 2007) that Th1 cells are associated with the stable gingivitis lesion while Th2 cells are associated with the progressive periodontitis lesion. Some studies did not agree and showed a mixed Th1 and Th2 responses in advanced periodontitis (Berglundh et al., 2002; Fujihashi et al., 1996; Prabhu et al., 1996).

Over all, the respective roles of Th1 and Th2 cells in human disease and periodontal disease remain inconclusive.

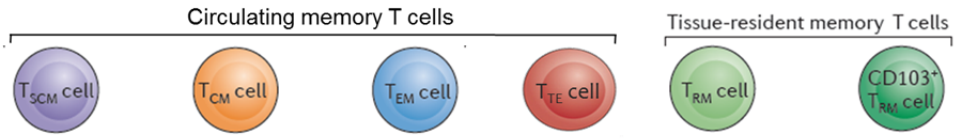
Other $CD4^+$ T cell subsets, the so-called Th17 and Treg (regulatory T cells), have been described. Both of which add complexity into the cytokine network. Th17 secrete a pro-inflammatory cytokine-IL-17 while Treg secrete IL-10 and TGF-β (Noack and Miossec, 2014). Treg functions opposite to Th17 cells, which inhibit tissue inflammation and maintaining self-tolerance(Awasthi and Kuchroo, 2009). Both Th17 and Treg were identified in periodontitis however, their physiological role in protection and disease are still unclear.

Memory T cells

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 (reviewed in (Sallusto et al., 1999)). Following antigen clearance, such as that in smallpox vaccination, >95% of the effector cells die while a small pool of T cells ultimately develops into long-lived memory T cells (Zhang et al., 2005). The memory T cells found in the blood can be divided into subsets based on the differential expression of markers of migration, CD62L and CCR7 (Sallusto et al., 1999). Those with high expression of CD62L and CCR7 are termed central memory T cells (T_{CM}) because they are enriched in the secondary lymphoid organs. By contrast, memory T cells lacking CD62L and CCR7 expression migrate between blood and non-lymphoid tissue, exhibiting rapid effector capabilities on stimulation and so are termed effector memory cells (T_{EM}). These T_{EM} can be differentiated into terminal effector memory T cells (T_{TE}) by IL-15 (Lugli et al., 2010). Studies in both mice and humans have led to identification of another subset of memory T cells which retain stem cell-like properties similar to hematopoietic stem cells and generate multiple subsets of memory T cells in vitro (Gattinoni et al., 2011; Zhang et al., 2005).These cells are known as stem cell memory T cells (T_{SCM}). T_{CM} and T_{SCM} express CD28 and CD95, while T_{EM} and T_{TE} express CD28 but no CD95.

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 (Ariotti et al., 2012; Di Meglio et al., 2011; Sathaliyawala et al., 2013). The mechanism of T_{RM} retention in tissues is not precisely known. T_{RM} 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 (Pauls et al., 2001). It

should be pointed out that CD103- T_{RM} also can be detected and localize to the same clusters that $CD103^{+}T_{RM}$ do (Wakim et al., 2010). Another key cell surface marker of T_{RM} is CD69. In addition to its association with recent activation, CD69 inhibits the function of sphingosine-1-phosphate receptor 1 (S1P1), resulting in retention of newly primed T cells in draining lymph nodes (Shiow et al., 2006). Therefore the CD69/S1P1 may play a role in inhibiting T_{RM} cell egress from tissue. This $CD103/CD69^{+}$ phenotype is not expressed among pathogen-specific memory CD8 T cells in blood (Mueller et al., 2013). It should be pointed that T_{RM} can be further classified as T_{RM} $CD103^{-}$ and T_{RM} $CD103^{+}$ (Farber et al., 2014).



CD45RA	+	-	-	+	-	-
CCR7	+	+	-	-	-	-
CD28	+	+	-	-	-	-
CD95	+	+	+	+	+	+
CD69	-	-	-	-	+	+
CD103	-	-	-	-	-	+
IL-2	+++	+++	++	+/-	+/-	+/-
IFN	+	++	+++	+++	+++	+++
TNF	+	++	+++	+++	+++	+++

Figure1. Schematic of human memory T cell heterogeneity in the blood and in tissues. Four circulating populations include stem cell memory T cells (T_{SCM}), central memory T cells (T_{CM}), effector memory cells (T_{EM}), and terminal effector memory T cells (T_{TE}). Two tissue populations include tissue-resident memory T (T_{RM}) cells with $CD103^{+}T_{RM}$ and $CD103^{-}T_{RM}$.

While circulating memory T cells provide efficient protection against systemic infections, their ability to deal with localized infections in the periphery is often limited. In a mouse model of viral infection, it was clearly demonstrated that T_{RM} provide superior protection against viral infection relative to the circulating memory T cells (Gebhardt et al., 2009; Jiang et al., 2012; Mackay et al., 2012). T_{RM} cells generated in skin and salivary glands after Vaccinia virus or LCMV infection, mediate potent protection from infection rechallenge even when T cell recirculation is pharmacologically inhibited (Hofmann and Pircher, 2011; Liu et al., 2009;

Teijaro et al., 2011). T_{RM} established in the vagina epithelial layer by exogenous chemokine treatment provide better protection against a lethal vagina HSV-2-challenge compared to circulating HSV-2-specific memory T cells (Shin and Iwasaki, 2012). Infection of mice with influenza virus leads to the generation of both resident and transient circulating memory T cells in the lungs; however, lung T_{RM} CD4 T cells and CD8 T cells show optimal protection against influenza challenge compared with circulating memory T cells (Teijaro et al., 2011; Turner et al., 2014; Wu et al., 2014). Collective evidence suggests that T_{RM} in peripheral tissues play a key role in mediating T cell–dependent protective immunity against microbial pathogens. Furthermore, these findings also suggest that peripheral blood immune response may differ from those at the tissue sites where they are needed.

While recent advances have been made on memory T cells from different mucosal tissues, most of these data have been derived from a mouse model which could be different from human immune response. Thus, we propose for the first time to comprehensively to investigate different memory T cell subsets in human healthy and periodontitis tissues. This proposed study will provide new insights into subsets of memory T cells in human periodontitis and their specific cytokine response.

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MATERIALS AND METHODS

Reagents

Monoclonal antibodies (mAbs) against the following proteins for flow cytometry: CD3 (SK7), CD4 (RPA-T4), CD8 (RPA-T8), CD 28 (CD28.2), CD95 (DX2), CD45RA (5H9), CCR7 (150503), CD69 (FN50), CD103 (Ber-ACT8) and IFN-γ (B27) were form BD Biosciences; IL-17A (eBio64CAP17) was from eBioscience. mAbs against the following proteins for immunohistochemistry analysis: CD3 (A0452) was from DAKO, CD4 (EPR6855) and CD103 (EPR4166(2)) were from Abcams and CD8 (1A5) was from Leica Biosystems.

Ethic statement

Ethical approval was obtained from the Ethics Committee Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2013-016). Written informed consent of all participating subjects was obtained prior to inclusion in the study.

Gingival tissue specimens

Gingival tissues were obtained from patients with severe chronic periodontitis and subjects with healthy gingiva. These specimens were collected from patients at the Periodontal Clinic and Oral Maxillofacial Surgery Clinic, Faculty of Dentistry, Chulalongkorn University. Each patient had at least 16 remaining teeth with no history of periodontal treatment for the past 6 months. Subjects with healthy gingiva showed no sign of gingival inflammation (no bleeding on probing, probing depth < 4 mm) and no clinical attachment loss or bone loss. Healthy gingival tissue were collected during crown-lengthening procedure for prosthetic or orthodontic reasons. Periodontitis tissues were collected from sites of extracted teeth that were irrational to treat (bleeding on probing, probing pocket depth > 6 mm and bone loss > 60% of the root). All subjects were in good general health, and had not taken antimicrobial or anti-inflammatory drugs within the previous 3 months.

Immunohistochemistry

Gingival tissues were fixed in 10% buffered formalin for a maximum of 24 h and subsequently embedded in paraffin. Microtome serial 4-micron-thick sections were cut and mounted on glass slides. Sections were deparaffinized. To inhibit endogenous peroxidase, the sections were incubated in 0.3% hydrogen peroxide solution for 20 min, and heated in 1 mM EDTA pH 8.0 at 95°C for 20 min for antigen retrieval.

To identify $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells, staining was performed on the sections via Polymer/HRP and DAB^+chromagen system (DAKO EnVisionTM G/2 Doublestain System). The samples were stained with the designated primary antibodies followed by Polymer/HRP, and then counterstained with hematoxylin.

Flow cytometric analysis of memory T cell subsets

Cells from healthy gingiva and periodontitis tissues were stained with mAbs against CD3, CD4, CD8, CD28, CD95, CD45RA and CCR7. T cells expressing $CD45RA^+$ $CCR7^+CD28^+CD95^-$ were defined as naïve T cells. Phenotypic markers of memory T cell subsets were characterized as follows:

1) $CD45RA^+CCR7^+CD28^+CD95^+$ cells were T_{SCM} cells

2) $CD45RA^-$ $CCR7^+CD28^+CD95^+$ cells were T_{CM} cells

3) $CD45RA^-$ $CCR7^-CD28^+CD95^+$ cells were T_{TM} cells

4) $CD45RA^-CCR7^-CD28^-CD95^+$ cells were T_{EM} cells

5) $CD45RA^+CCR7^-CD28^-CD95^+$ cells were T_{TE} cells

To identify T_{RM} cell markers; CD69 and CD103, cells were stained with mAbs against CD3, CD4, CD8, CD69 and CD103. $CD69^-$ memory T cells were characterized as recirculating memory T cells, while $CD69^+$ memory T cells were characterized as gingiva-resident memory T cells. Flow cytometry analysis was performed using 12-color flow cytometry, FACSCelesta (BD Biosciences).

Intracellular staining analysis of IL-17 and IFN-γ

Gingival cells were stimulated with Staphylococcal enterotoxin (SEB) (4 μg/ml) and gingival cells cultured in medium served as negative control. After 2 h of stimulation, Golgiplug was added to inhibit cytokine secretion and the cell cultures were further incubated overnight. The cells were washed and stained with a panel of antibodies including anti-CD3, anti-CD4, anti-CD8 and anti-CD103 mAbs. The stained cells were

fixed/permeabilized and intracellular cytokines were stained with mAbs against IL-17 and IFN-γ. Finally, stained cells were analyzed by 12-color flow cytometer.

Statistical analysis

The data were analyzed using SPSS 22.0 for Windows (SPSS). Results were shown as mean ± SEM. Comparisons of one variable between two groups were analyzed using the Mann-Whitney *U* test. Comparisons between two variables in one group were analyzed using the Wilcoxon signed-rank test. Two-tailed *p* values <0.05 were considered statistically significant.

RESULTS

Human memory T cells in healthy gingiva

Immunohistochemical staining was used to investigate the presence and anatomical location of T cells in healthy gingiva. Fig. 2 showed that $CD3^+$ T cells formed clusters close to capillary opening, scattered throughout connective tissue. Some were detected in the epithelial layer. Dense $CD3^+$ T cell clusters were consistently observed at the bottom of the healthy gingival sulcus adjacent to the junctional epithelium. $CD4^+$, $CD8^+$ and $CD103^+$ T cells were also detected in the same locations.

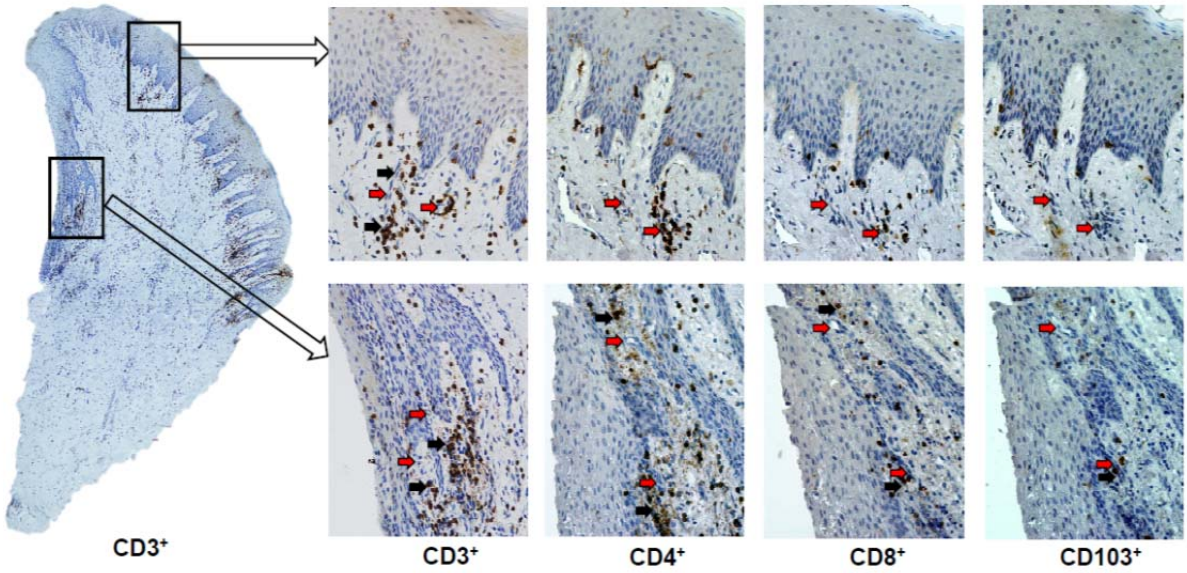

Figure 2. Anatomical compartmental location of T cells in healthy gingiva. Representative immunostaining of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ T cells from 7 different healthy individuals. Black arrows indicate T cells. Red arrows indicate capillary in the connective tissue.

Most of the T cells isolated from healthy gingiva were memory T cells (Fig. 3A). The percentage of $CD4^+$ memory T cells in healthy gingiva was consistently higher compared with $CD8^+$ memory T cells (61.52%±1.47 vs 38.48%±1.47, P <0.001, (Fig. 3B).

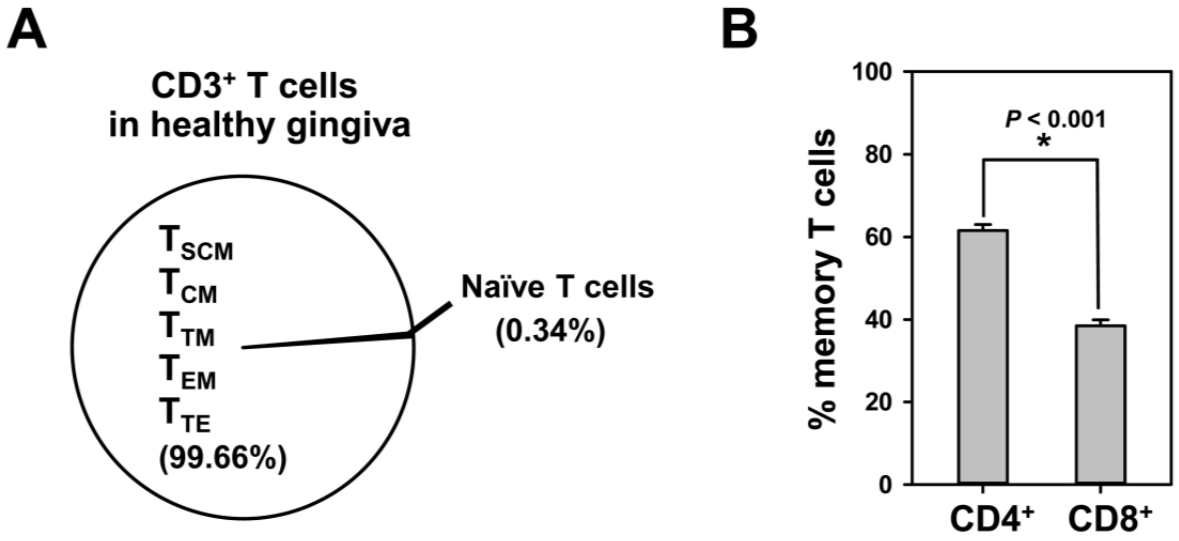

Figure 3. (A) Percentage distribution of memory T cells vs naïve T cells in healthy gingiva. (B) Frequency of $CD4^+$ vs $CD8^+$ memory T cells in healthy gingiva (n=9).

We next analyzed the expression of CD69 and CD103, surface markers commonly used to identify T_{RM} cells. T cells expressing CD69 were defined as gingiva-resident memory T cells whereas those not expressing CD69 were defined as recirculating memory T cells. Healthy gingiva contained both $CD69^-$ recirculating memory T cells (56.26%±4.31) and $CD69^+$ gingiva-resident memory T cells (43.74%±4.31; P = 0.203) (Fig. 4). Most of $CD69^-$ and $CD69^+$ memory T cell populations were $CD4^+$ cells and were CD103 negative ($CD4^+CD69^-CD103^-$ T cell frequency = 34.21%±3.17; $CD4^+CD69^+CD103^-$ T cell frequency = 26.68%±2.96). Only a small proportion of memory T cells expressed CD103, which was mostly detected on $CD8^+$ T cells.

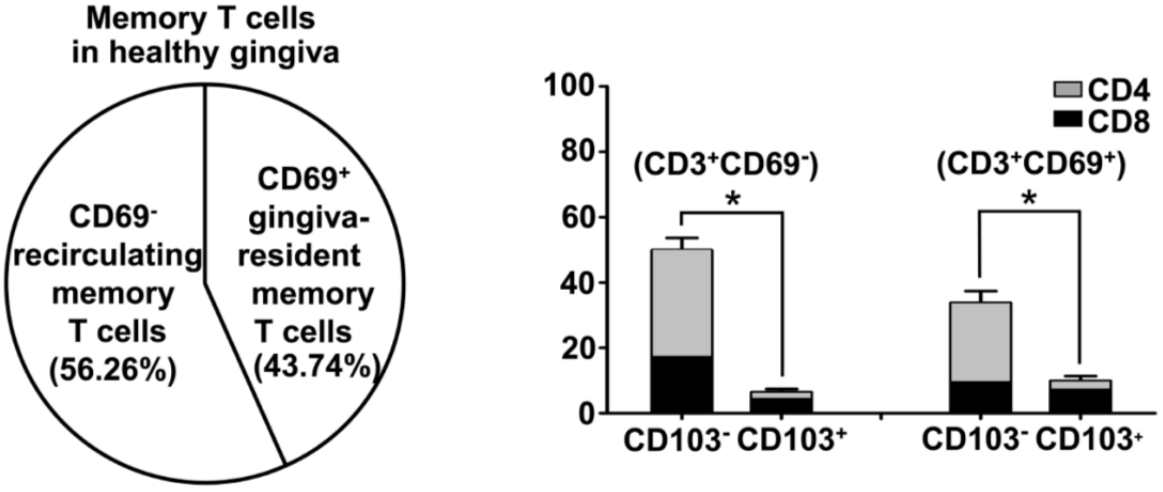

Figure 4. Percentage distribution of CD69- recirculating memory T cells and CD69+ gingiva-resident memory T cells. Mean ± SE of n=9 are shown.

We next characterized the composition of the memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in the recirculating and gingiva-resident memory T cell populations. Fig. 5A showed that $CD45RA^{-}CCR7^{-}CD28^{+}CD95^{+}$ T_{TM} cells (73.17 %±3.71) and $CD45RA^{-}CCR7^{+}CD28^{+}CD95^{+}$ T_{CM} cells (22.17%±3.13) composed the majority of $CD4^{+}CD69^{-}CD103^{-}$ recirculating memory T cells. The percentages of T_{SCM}, T_{EM}, and T_{TE} cells were negligible (0.1-2%). Within the $CD8^{+}CD69^{-}$ $CD103^{-}$ recirculating memory T cells, the T_{TM} cells comprised 48.42%±4.43, followed by $CD45RA^{-}CCR7^{-}CD28^{-}CD95^{+}$ T_{EM} (29.10%±4.53), $CD45RA^{+}CCR7^{-}CD28^{-}CD95^{+}$ T_{TE} (11.53%±2.43), $CD45RA^{+}CCR7^{+}CD28^{+}CD95^{+}$ T_{SCM} (5.73 %±0.99), and T_{CM} cells (5.16%±0.62). The proportion of $CD69^{-}CD103^{+}$ recirculating population was minimal and their memory T cell subset compositions was similar to those in the $CD69^{-}CD103^{-}$ recirculating population (Fig. 5B).

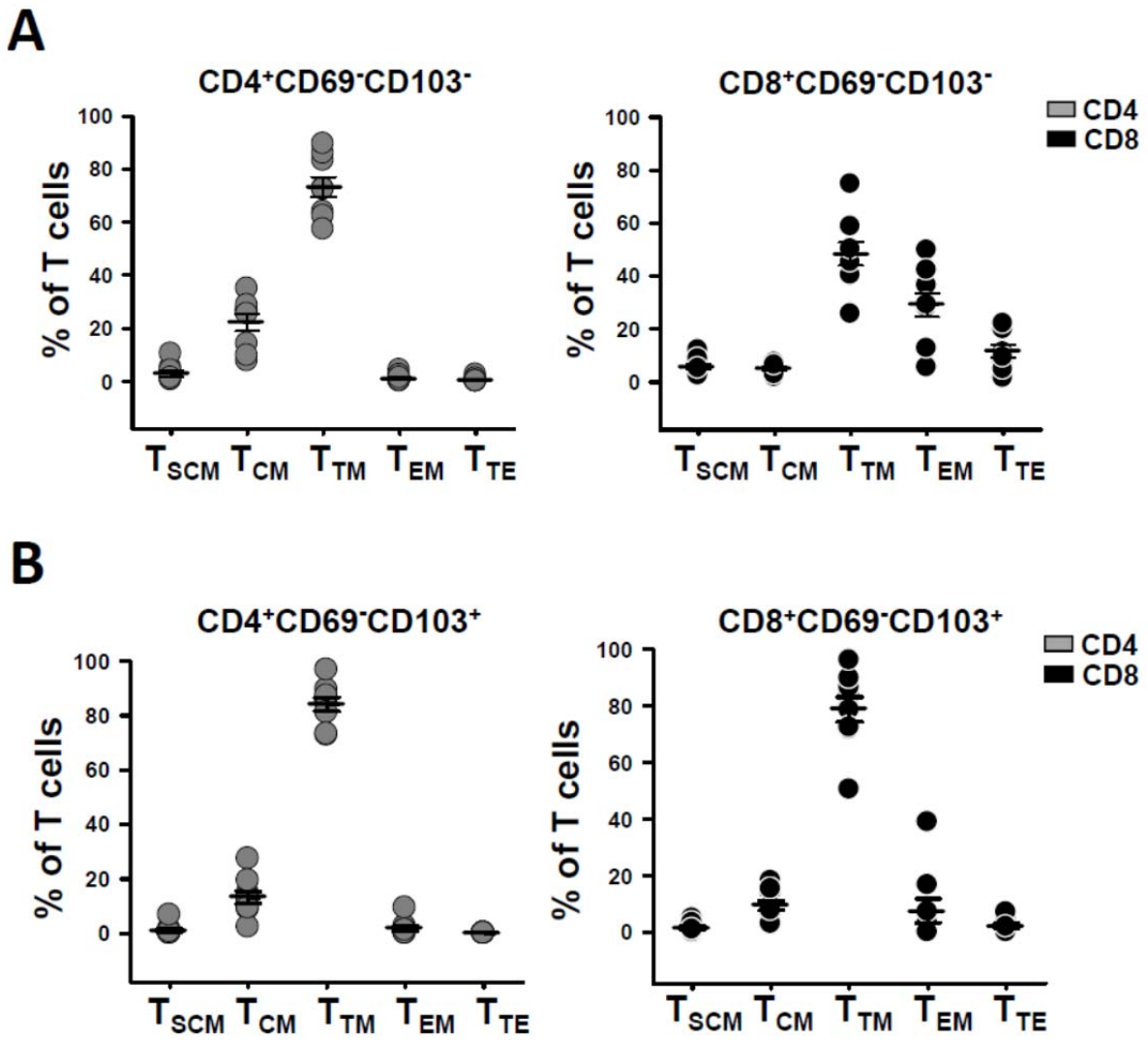

Figure 5. The composition of memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in (A) $CD4^+CD69^-CD103^-$ and $CD8^+CD69^-CD103^-$, (B) $CD4^+CD69^-CD103^+$ and $CD8^+CD69^-CD103^+$ Each data point represents the value of an individual sample and means ± SE are shown (n = 9).

The composition of the memory T cell subsets of the $CD69^+$ gingiva-resident memory T cells was evaluated. T_{TM} and T_{CM} cells made up 77.55%±1.87 and 19.72%±2.03, respectively, of the $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells. The percentages of T_{SCM}, T_{EM}, and T_{TE} cells were negligible (0.14 - 2.15%). Analysis of the $CD8^+CD69^+CD103^-$ gingiva-resident memory T cells indicated that the T_{TM} cells constituted 60.73%±3.69, followed by T_{TE} (22.82%±3.71), T_{EM} (6.21%±1.23), T_{CM} (5.39%±1.44) and T_{SCM} cells (4.85%±1.01) (Fig. 6A).

Only a small population of the $CD69^+$ gingiva-resident T cells expressed CD103. In the $CD4^+CD69^+CD103^+$ gingiva-resident memory T cells, most were T_{TM} cells (83.14%), followed by T_{CM} (13.39%±2.00), T_{EM} (3.169%±2.15), and T_{SCM} cells (0.05%±0.04). Among the $CD8^+$ $CD69^+CD103^+$ gingiva-resident memory T cells, the majority were T_{TM} cells (68.01%±4.18), followed by T_{EM} (15.60%±3.41), T_{TE} (10.17%±2.32), T_{CM} (5.53%±1.2) and T_{SCM} cells (0.69%±0.27) (Fig. 6B).

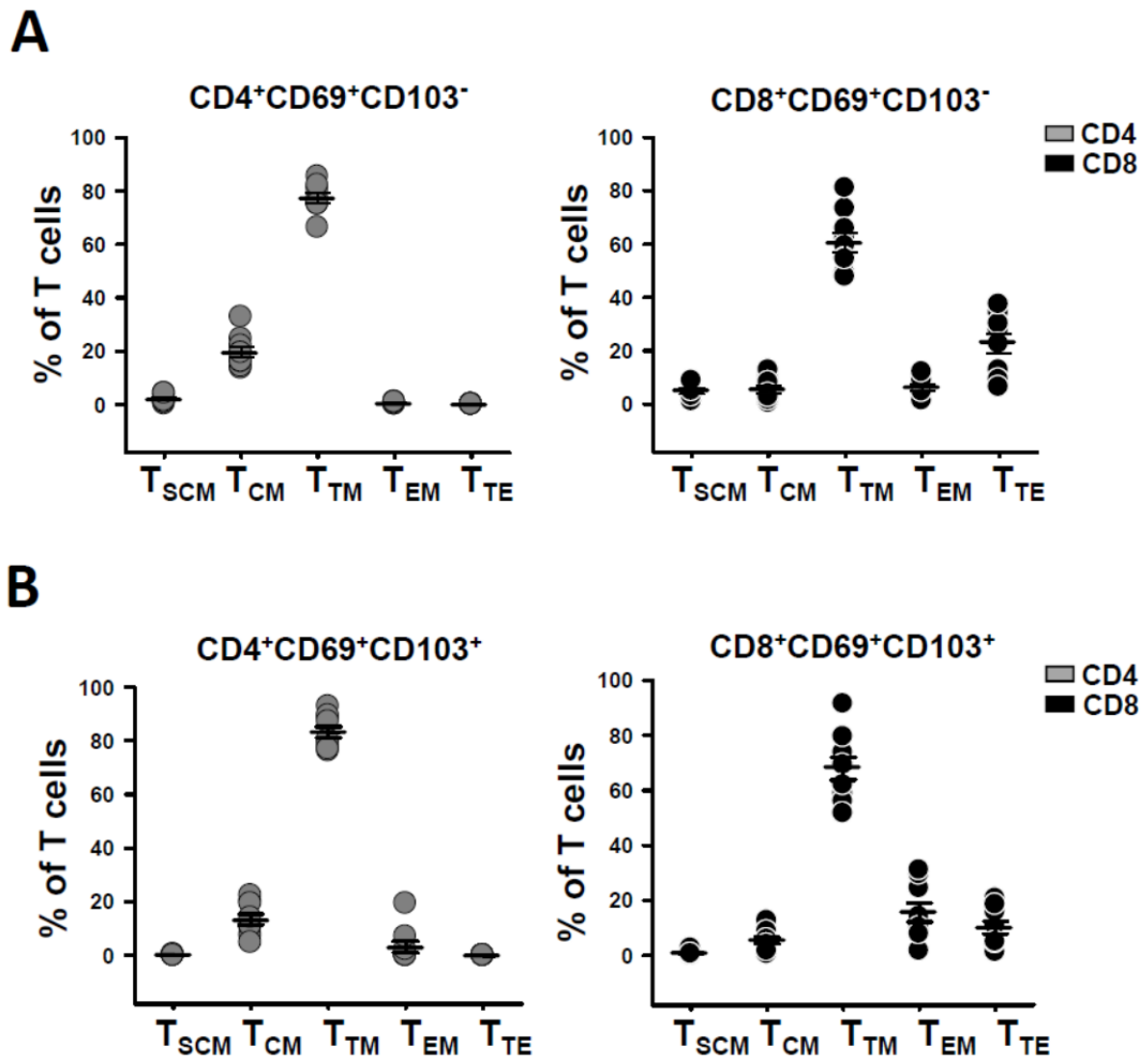

Figure 6. The composition of memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in (A) $CD4^+CD69^-CD103^+$ and $CD8^+CD69^-CD103^+$, (D) $CD4^+CD69^+CD103^-$ and $CD8^+CD69^+CD103^-$, (B) $CD4^+CD69^+CD103^+$ and $CD8^+CD69^+CD103^+$. Each data point represents the value of an individual sample and means ± SE are shown (n = 9).

Increased accumulation of memory T cells in periodontitis tissues

Immunohistochemical staining of periodontitis tissues showed an increase of $CD3^+$ T cells at the base of the periodontal pocket area and scattered throughout the connective tissue, especially apically toward the advancing front of the lesion. Increased $CD3^+$ T cells in the epithelial layer were not observed (Fig. 7). Numerous $CD4^+$, $CD8^+$ and $CD103^+$ T cells were also detected in the connective tissue.

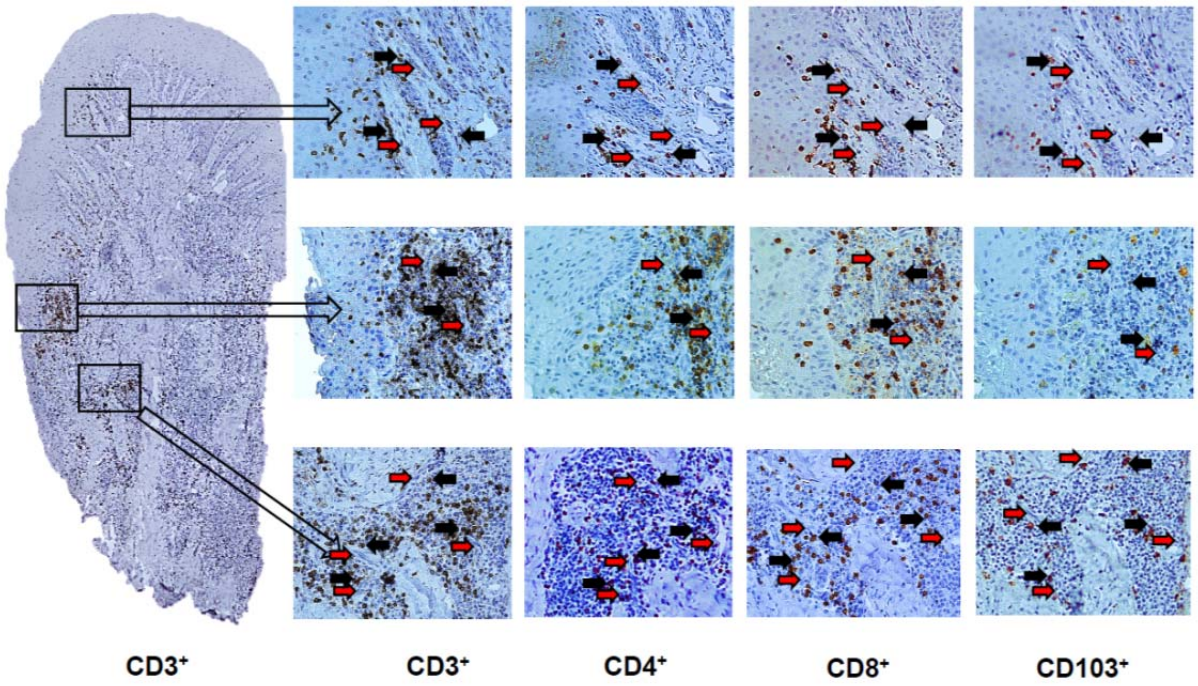

Figure 7. Anatomical compartmental location of T cells in periodontitis tissue. Representative immunostaining of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ T cells from 11 different periodontitis subjects. Black arrows indicate $CD3^+$ T cells. Red arrows indicate capillary in the connective tissue.

Similar to healthy gingiva, most of the T cells isolated from periodontitis tissues were memory T cells (Fig 8A) and a higher proportion of $CD4^+$ compared with $CD8^+$ (65.03%±5.24 vs 34.97%±5.24; $P < 0.05$) memory T cells was observed (Fig. 8B).

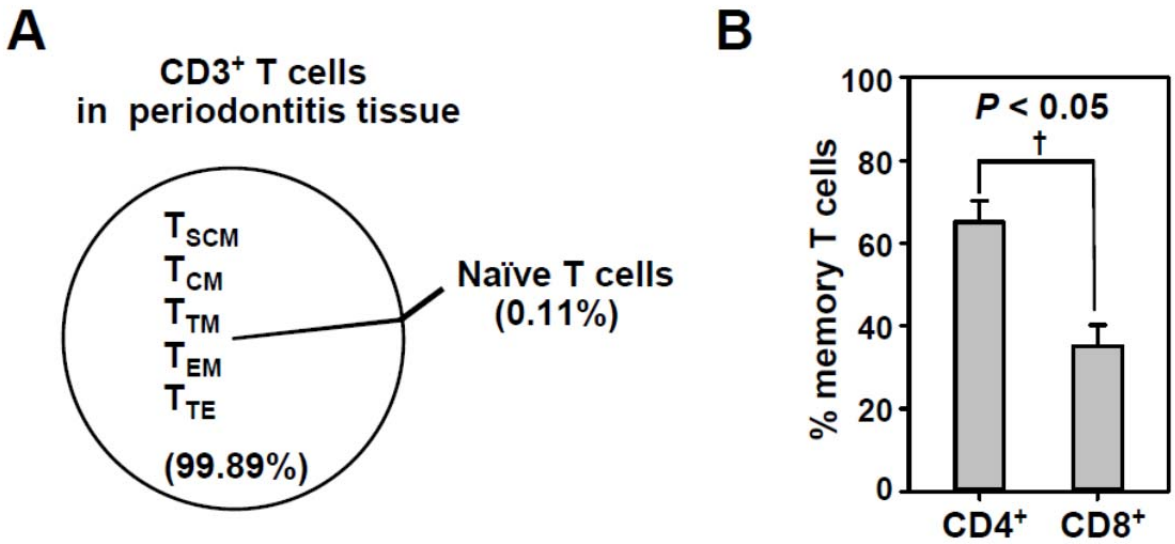

Figure 8. (A) Percentage distribution of memory T cells vs naïve T cells in periodontits tissues. (B) Frequency of $CD4^+$ vs $CD8^+$ memory T cells in periodontits tissues (n=9).

Interestingly, the proportion of $CD4^+CD69^-CD103^-$ recirculating memory T cells was significantly lower in periodontitis tissues compared with healthy gingiva (32.40%±3.03 vs 22.33%±2.54; *P* = 0.037). In contrast we observed an increased proportion of $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells compared with healthy gingiva (36.85%±3.22 vs 24.11%±2.70; *P* = 0.017) (Figs 9, and 4).

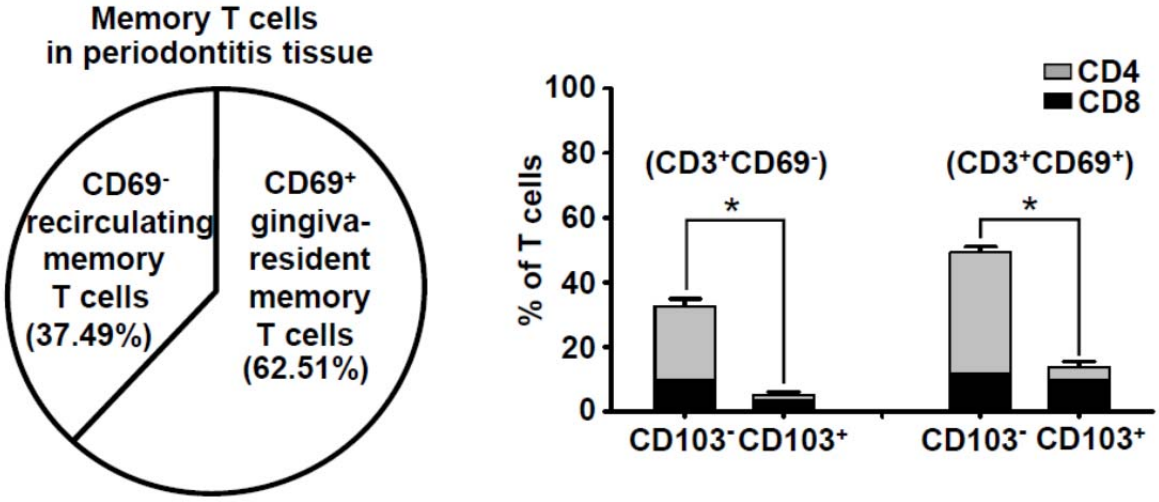

Figure 9. Composition of memory T cell subsets in periodontitis tissue**.** (A) Percentage distribution of $CD69^-$ recirculating memory T cells and $CD69^+$ gingiva-resident memory T cells. Means ± SE of n = 4 are shown.

The composition of the memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in periodontitis tissues were similar to those in healthy gingiva. $CD4^+$ and $CD8^+$ T_{TM} cells (67.40% - 91.91%) constituted the major subset in both recirculating and gingiva-resident memory T cell populations (Figs. 10B, 10B, 11A and 11B).

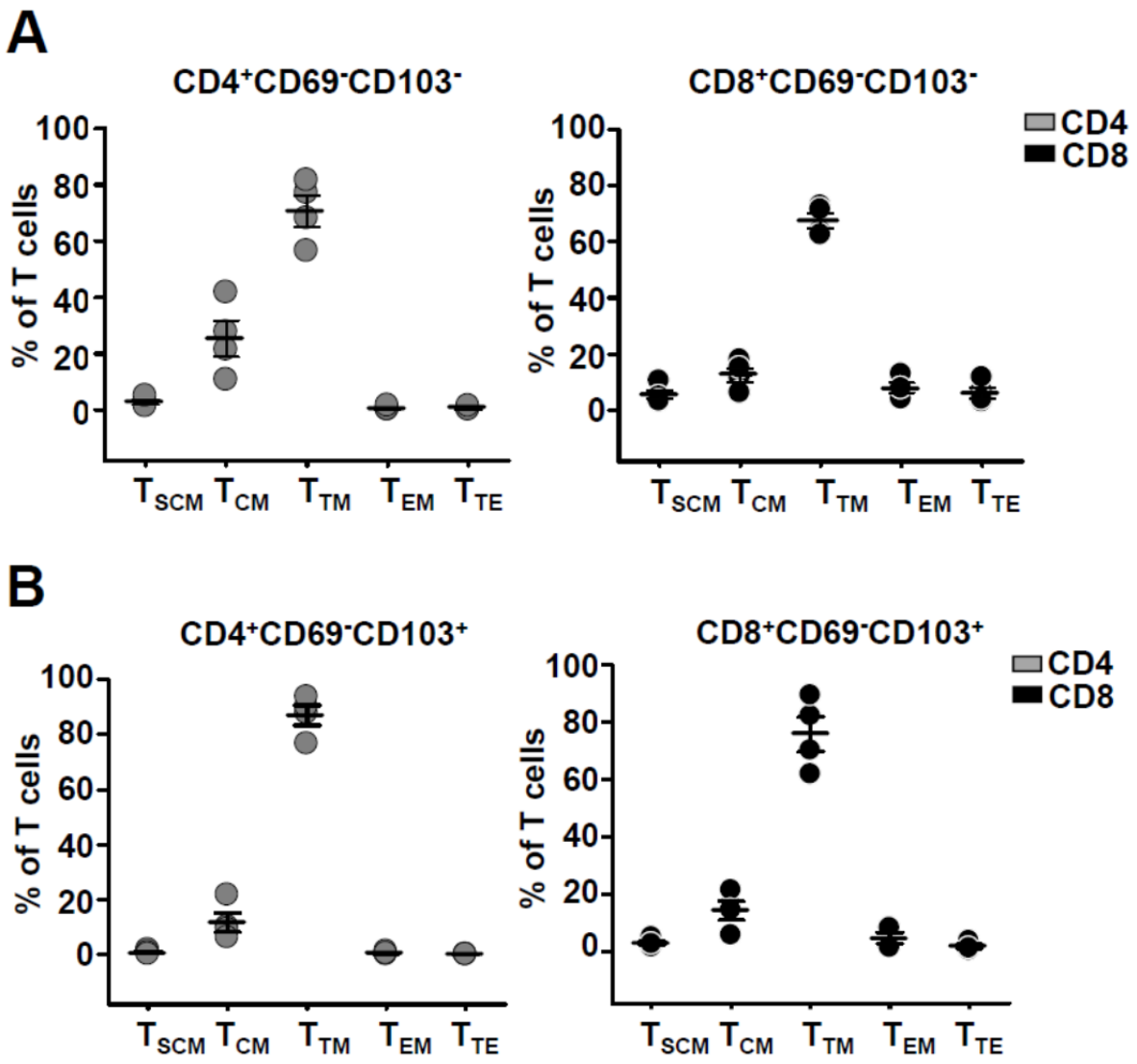

Figure 10. The composition of memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in (A) $CD4^+CD69^-CD103^-$ and $CD8^+CD69^-CD103^-$, (B) $CD4^+CD69^-CD103^+$ and $CD8^+CD69^-CD103^+$. Each data point represents the value of an individual sample and means ± SE are shown (n = 4).

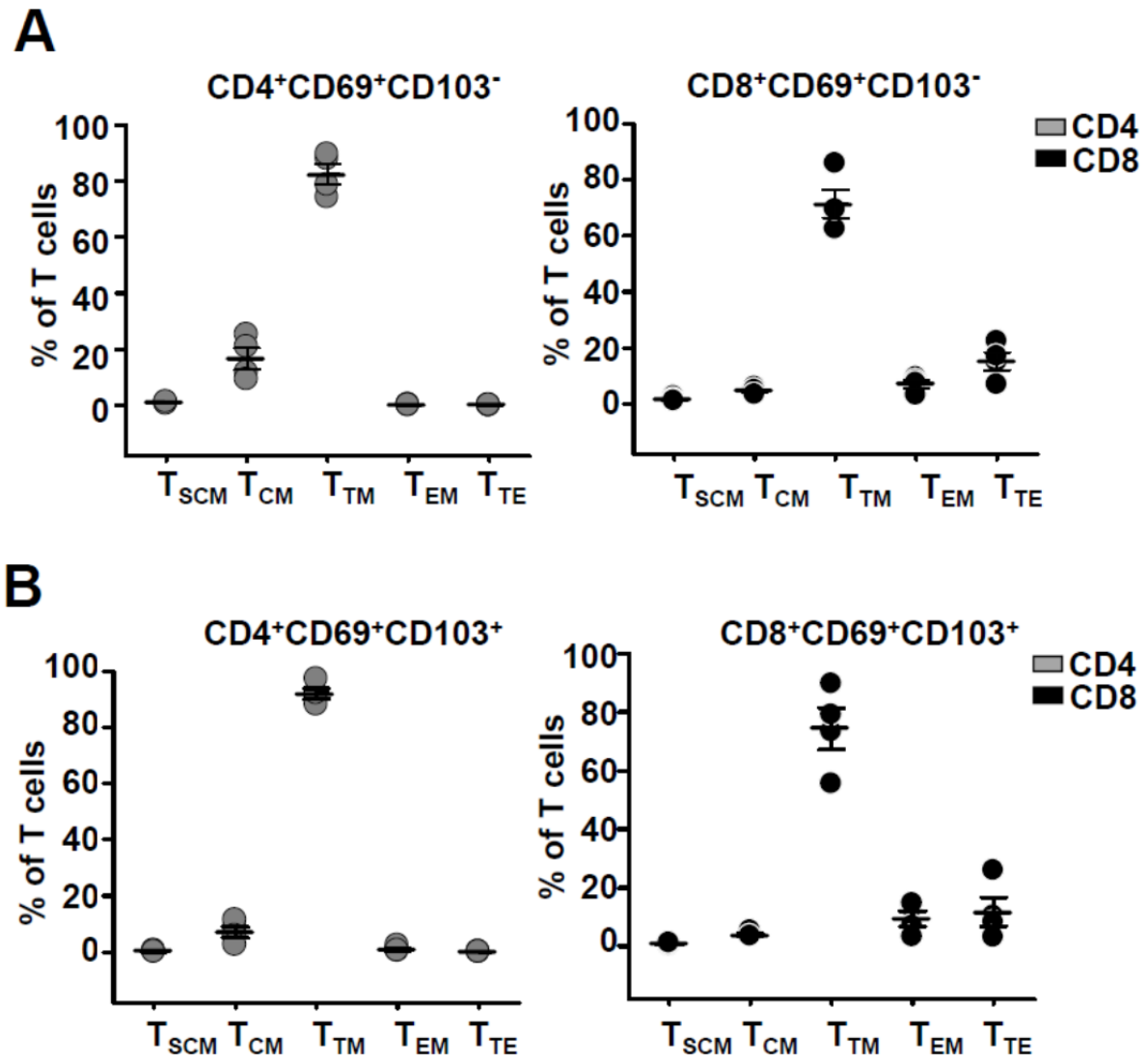

Figure 11. The composition of memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in (A) $CD4^+CD69^+CD103^-$ and $CD8^+CD69^+CD103^-$, (B) $CD4^+CD69^+CD103^+$ and $CD8^+CD69^+CD103^+$. Each data point represents the value of an individual sample and means ± SE are shown (n = 4).

IL-17 and IFN-γ responses from memory T cells isolated from periodontitis tissues

The small number of T cells that could be isolated from healthy gingiva limited the study of IL-17 and IFN-γ responses. Only single healthy tissue sample shown in Fig 12. Memory T cells produced both IL-17 and IFN-γ, similar to those form periodontitis tissues.

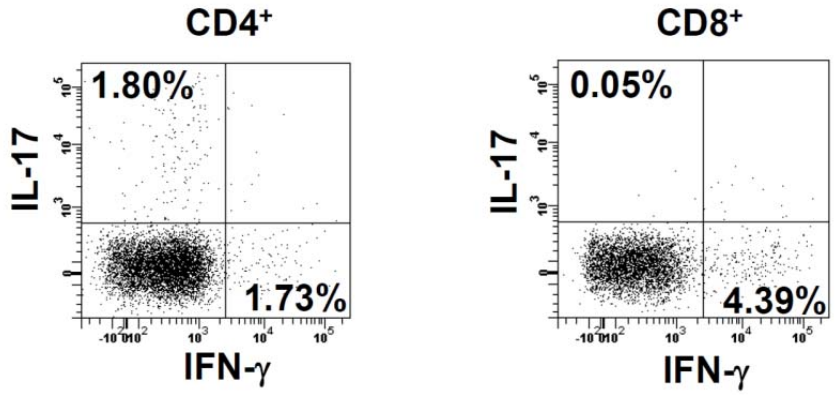

Figure 12. Expression of IL-17 and IFN-γ of $CD4^+$ and $CD8^+$ memory T cells isolated from single healthy gingival tissue sample.

Cytokine responses from T cells derived from periodontitis tissues are shown in Fig. 13. Memory T cells produced both IL-17 and IFN-γ. $CD4^+$ memory T cells predominant produced IL-17 alone (frequency = 1.88%±0.52) and IFN-γ alone (frequency = 3.42%±1.82). The frequency of $CD4^+$ T cells that produced IL-17 plus IFN-γ was negligible. Unlike $CD4^+$ memory T cells, $CD8^+$ memory T cells mostly produced IFN-γ alone (frequency = 8.51%±1.41).

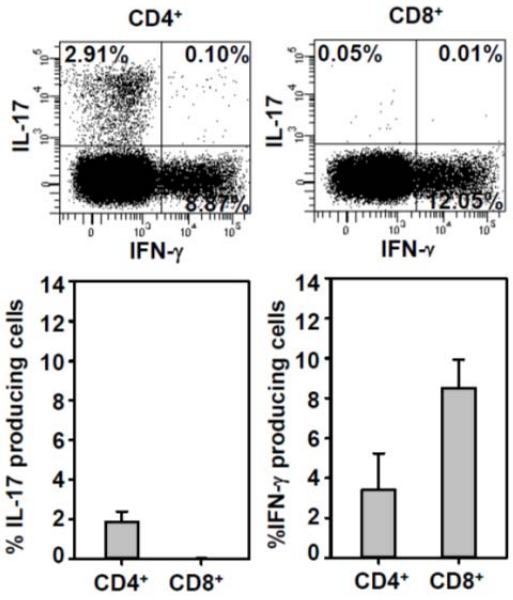

Figure 13. Expression of IL-17 and IFN-γ of $CD4^+$ and $CD8^+$ memory T cells isolated from periodontitis tissue. Representative of flow cytometry plot of $CD4^+$ and $CD8^+$ memory T cells expressing IL-17 and IFN-γ and frequencies of IL-17 and IFN-γ production from $CD4^+$ and $CD8^+$ memory T cells. Mean ± SE of n = 4 are shown.

Measurement of IL-17 and IFN-γ production in recirculating vs gingiva-resident memory T cells based on CD69 expression was not possible because *in vitro* stimulation with SEB leads to CD69 expression on most T cells (Pichyangkul S et al., 2015). We next compared the cytokine response between the memory T cell $CD103^+$ and $CD103^-$ subsets. We found that the frequency of $CD4^+$ $CD103^+$ memory T cells that produced IFN-γ was 4.4 fold higher than that of $CD4^+CD103^-$ memory T cells however, the differences did not reach statistical significance (12.87%±4.82 vs 2.92%±1.55; P = 0.06) (Fig. 14A). The frequency of IL-17 producing cells between $CD4^+$ $CD103^+$ and $CD4^+CD103^-$ memory T cells was similar (1.49%±0.25 vs 1.92%±0.51). A significant difference in the frequency of IFN-γ producing cells between $CD8^+CD103^+$ and $CD8^+$CD 103^- memory T cells was observed (11.40%±1.02 vs 7.56%±1.07 ; P = 0.029) (Fig 13B).

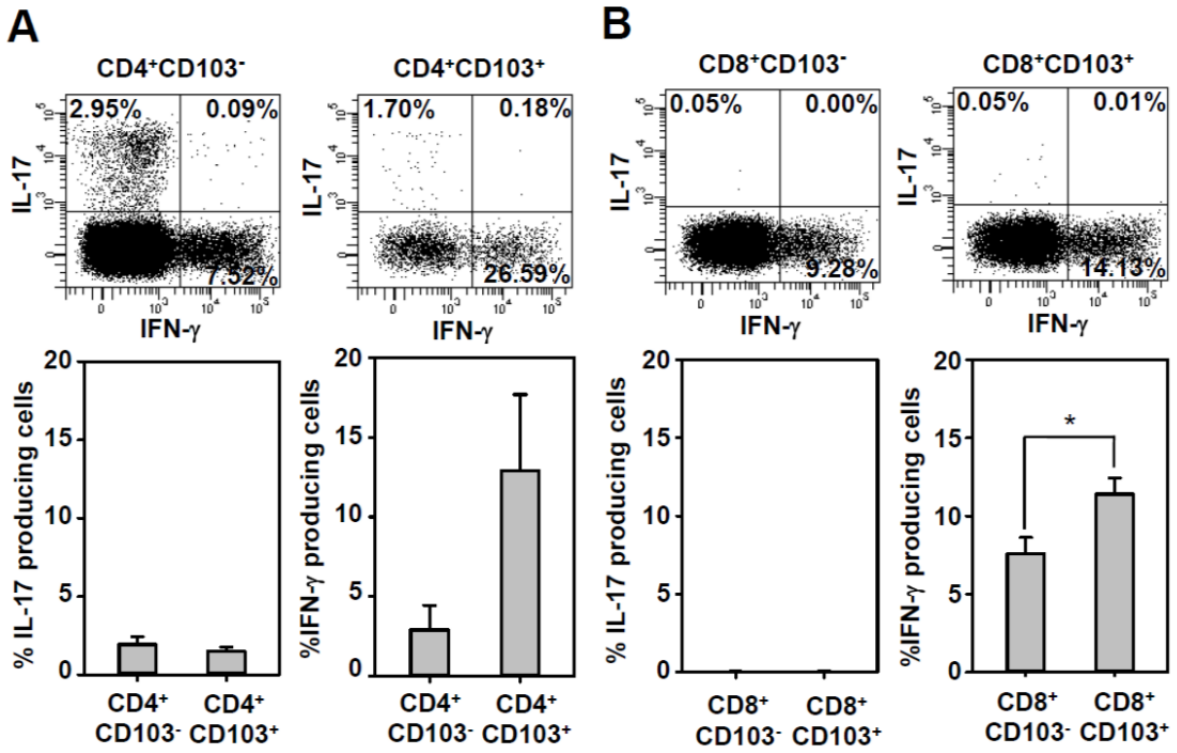

Figure 14. Expression of IL-17 and IFN-γ of $CD4^+$ and $CD8^+$ memory T cells isolated from periodontitis tissue. (A) Representative of flow cytometry plot of $CD4^+$ and $CD8^+$ memory T cells expressing IL-17 and IFN-γ and frequencies of IL-17 and IFN-γproduction from $CD4^+$ and $CD8^+$ memory T cells. Mean ± SE of n = 4 are shown. (B) Representative of flow cytometry plot of $CD4^+CD103^-$ and $CD4^+CD103^+$ memory T cells expressing IL-17 and IFN-γ and frequencies of IL-17 and IFN-γproduction from $CD4^+CD103^-$ and $CD4^+CD103^+$ memory T cells. Mean ± SE of n = 4 are shown. (C) Representative of flow cytometry plot of $CD8^+CD103^-$ and $CD8^+CD103^+$ memory T cells expressing IL-17 and IFN-γand frequencies of IL-17 and IFN-γ

production from $CD8^+CD103^-$ and $CD8^+CD103^+$ memory T cells. Mean ± SE of n = 4 are shown.

Granzyme B expression was detected in control unstimulated cultures and did not increase after SEB stimulation (Fig 15).

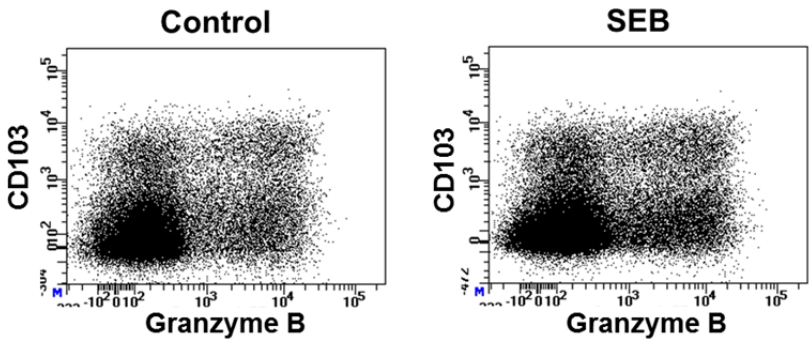

Figure 15. Expression of granzyme B of $CD3^+$ memory T cells isolated from periodontitis tissue.

DISCUSSION

There is a lack of reports concerning T cells in healthy gingiva. Using immunohistochemical staining and flow cytometry, we showed that, like other human mucosal tissues, most T cells isolated from healthy gingiva had a memory phenotype. The two major populations of these memory T cells are recirculating and gingiva-resident memory T cells, characterized by the expression of CD69, a well-known T_{RM} retention marker. Gingival memory T cells formed clusters scattered in the connective tissue, preferentially subjacent to the junctional epithelium, a strategic area vulnerable to subgingival plaque microbiome. Our findings highlight the possible role of memory T cells in the healthy gingiva immune surveillance network.

$CD4^+$ memory T cells with a T_{TM} cell phenotype were the major subset in both $CD69^-$ recirculating and $CD69^+$ gingiva-resident memory T cells. $CD8^+$ memory T cells in healthy gingiva represented a minority subset and had T_{TM} cell phenotype. These results were different from other studies showing that tissue T cells had a T_{EM} cell phenotype (Farber DL et al., 2014; Masopust D et al., 2015). These discrepancies may result from using different cell surface markers for memory T cell phenotype identification. While most studies used $CCR7^-$ to define the T_{EM} cell phenotype, we included two additional markers, anti-CD28 and anti-

CD95 mAbs in the antibody cocktail, allowing subset differentiation between T_{TM} cells ($CCR7^-$ $CD28^+CD95^+$) and T_{EM} cells ($CCR7^-CD28^-$CD95+). T_{TM} cells were first identified in blood of healthy individuals and has been suggested that they are more differentiated compared with T_{CM} cells but not as fully differentiated as T_{EM} cells (Fritsch RD et al., 2005; Okada R et al 2008.

Immunohistochemical staining of periodontitis tissues revealed the increase of memory T cell clusters at the base of the periodontal pocket and scattered throughout the gingiva, especially apically toward the advancing front of the lesion. Similar to healthy gingiva, $CD4^+$ T_{TM} cells also comprised the major subset in recirculating and gingiva-resident memory T cell populations. Interestingly, we detected a significant increase in the proportion of $CD4^+$ $CD69^+CD103^-$ gingiva-resident memory T cells in periodontitis tissues compared with healthy gingiva. This increase could result from a selective expansion of $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells that preexist in healthy gingiva. Alternatively, the expansion of these cells may result from the preexisting $CD4^+CD69^+CD103^-$ memory T cells combined with some of recirculating $CD4^+CD69^-$ $CD103^-$ memory T cells that become activated and express CD69 during the course of disease (CD69 expression in this case, represents an early T cell activation marker).

$CD4^+$ memory T cells isolated from periodontitis tissues produced either IL-17 or IFN-γ while $CD8^+$ memory T cells produced only IFN-γ These findings agree with recent observations suggesting that the major source of IL-17 in periodontitis is $CD4^+$ memory T cells (Dutzan N et al., 2016). $CD103^+$ memory T cells express higher levels of cytokines compared with $CD103^-$ memory T cells (Park and Kupper, 2015). In this study, we did not observe the differences in the magnitude of IL-17 and IFN-γ responses between $CD4^+CD103^+$ and $CD4^+CD103^-$ memory T cells. However, $CD8^+CD103^+$ memory T cells showed a significant higher IFN-γ response compared with $CD8^+CD103^-$ memory T cells.

Due to technical limitations, the number of T cells isolated from healthy gingiva was too low which limited cytokine investigation. However, data from single healthy tissue sample showed that memory T cells produced both IL-17 and IFN-γ, similar to those from periodontitis tissues (Supplementary Fig. 1). Our observations confirm recent studies describing that $CD4^+$ memory T cells from healthy gingiva produced IL-17 and IFN-γ (Dutzan N et al., 2016; 2017).

The role of IL-17 and IFN-γ in pathogenesis of periodontitis has been well described. IL-17 has been detected in periodontitis and is responsible for tissue damage and inflammatory bone loss (Eskan MA et al., 2012; Moutsopoulos NM et al., 2014). High expression of IFN-γ has been consistently detected in human periodontitis tissues (Dutzan N et al., 2009; Chen Xt et al., 2016). In mice that lack an IFN-γ response, the animals develop

less gingival tissue damage and bone loss following *A. actinomycetemcomitans* infection (Garlet GP et al., 2008).

Several evidences also point out tissue-protective and immune regulatory functions of IL-17 and IFN-γ in maintaining immune homeostasis. IL-17 stimulates antimicrobial peptide production by gut epithelial cells and maintain epithelial cell tight junctions at steady state (Weaver CT et al., 2013). IL-17 also promotes PMN migration across epithelium via IL-17-mediated IL-8 production (Takahashi N et al., 2011). The role of IFN-γ in maintaining immune homeostasis is less well described. A recent study suggests that IFN-γ regulates homeostasis in healthy tissue by up-regulation of suppressor-of-cytokine-2 protein expressed by tissue phagocytes (Nirschl CJ et al., 2017). Moreover, we previously described that IFN-γ induces indoleamine 2, 3-dioxygenase production from gingival fibroblast cells (Mahanonda R et al., 2008). This immune suppressing enzyme has been suggested to be a crucial mediator in maintaining gut homeostasis (Ciorba MA, 2013). The ability of IFN-γ to upregulate adhesion molecule VCAM-1 on endothelial cells (Schenkel JM et al., 2014) and ICAM-1 on gingival fibroblasts (Mahanonda R et al., 2008) may be responsible for the recruitment and retention of recirculating memory T cells observed in healthy gingiva. Collectively, these data suggest that IL-17 and IFN-γ responses, especially at low levels may have a beneficial role in supporting gingival tissue homeostasis and controlling tissue inflammation.

It should be pointed out that the current study was conducted using small sample size, limiting conclusions to some degree Another limitation is that we did not determine the frequency of regulatory T (T_{REG}) cells in healthy gingiva and periodontitis tissues. The function of T_{REG} cells has been demonstrated to counter-balance inflammatory responses (Smigiel KS et al., 2014). A better understanding of how T_{REG} cells may be involved in immune homeostasis and inflammation in human gingiva is required.

Classic notions regarding the crucial role of innate cells (junctional epithelium and PMN) in maintaining gingiva health have been long held. Our findings reveal that memory T cells may be involved in the healthy gingiva immune surveillance network and together with the innate arm of immune defense could maintain a homeostatic relationship with subgingival plaque microbiome. Microbial imbalance could damage the epithelial barrier allowing large amounts of bacteria and their antigens to gain access to the connective tissue. These antigens provoke uncontrolled T cell activation and cytokine production leading to tissue damage and bone loss. Further investigation to characterize memory T cell antigen specificity, the role of subgingival plaque bacteria, and specific tissue signals that promote the localization of recirculating and gingiva-resident memory T cells will provide additional insights into how the gingival immune response operates in health and disease.

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กิจกรรมอื่นที่เกี่ยวข้อง

1. ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

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2. ผลงานวิจัยอื่นๆ

    กำลังดำเนินงานวิจัยเรื่อง ปาก : ประตูสู่สุขภาพ

3. การนำผลงานไปใช้ประโยชน์

4. จำนวนและรายละเอียดการได้รับเชิญไปเป็นวิทยากร

    1. FDI 2015 Annual World Dental Congress กรุงเทพ ประเทศไทย กันยายน 2558
    2. งานประชุมวิชาการ 11th Asian Pacific Society of Periodontology Meeting ณ ประเทศอินโดนีเซีย ตุลาคม 2558
    3. การประชุมวิชาการ ทันตแพทยสมาคมแห่งประเทศไทย ในพระบรมราชูปถัมภ์ ธันวาคม 2558
    4. งานประชุมวิชาการ First JSDEI Symposium “Share and Exchange the Latest Findings on the Two-Way Relationship between Diabetes and Oral Health” ประเทศสิงคโปร์ มกราคม 2559

5. งานประชุมวิชาการ “งานประชุมราชวิทยาลัยทันตแพทย์แห่งประเทศไทย” กรุงเทพฯ กันยายน 2559

5. การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆ ทั้งในและต่างประเทศ

ได้รับเกียรติจากนักวิจัยที่มีชื่อเสียงระดับโลก Prof.Niklaus P. Lang มาบรรยายให้ความรู้ต่อทันตแพทย์ คณาจารย์ นักวิจัย นิสิตหลังปริญญา และผู้ช่วยทันตแพทย์ ที่จะได้รับความรู้และวิทยาการใหม่ๆ เกี่ยวกับงานทันตกรรมรากเทียม และยังช่วยในการเขียนงานวิจัยช่วยแนะนำ ติชม เพื่อต่อยอดพัฒนาความรู้ต่อไป

6. การเชื่อมโยงทางวิชาการกับนักวิชาการภายในสถาบันเดียวกัน

ร่วมกับทีมวิจัยของภาควิชาปริทันตวิทยา รวมทั้ง อาจารย์ของภาควิชาจุลชีววิทยา อาจารย์ของภาควิชาทันตพยาธิวิทยา อาจารย์ของภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ จัดทำชุดโครงการวิจัยเรื่อง ปาก : ประตูสู่สุขภาพ ขอรับการสนับสนุนจากจุฬาฯ

7. รางวัลที่ได้รับ

รางวัล Impact factor สูงสุดอันดับ 2 จากคณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปี 2559 ผลงานวิจัยเรื่อง Human Memory B Cells in Healthy Gingiva, Gingivitis, and Periodontitis

ภาคผนวก

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ORIGINAL ARTICLE

Memory T cell subsets in healthy gingiva and periodontitis tissues

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One-sentence summary: Our findings suggest that recirculating and gingiva-resident memory T cells could represent an important part of the immune surveillance network in the gingival connective tissue, maintaining immune homeostasis.

Abstract

Background: In the gingival sulcus, effective and balanced innate and adaptive immune responses against subgingival plaque microbiome are crucial to maintain immune homeostasis. In this study, we investigated the memory T cell subsets in healthy gingiva and periodontitis tissues.

Methods: Anatomical localization of T cells ($CD3^+$, $CD4^+$, and $CD8^+$) in healthy gingiva and periodontitis tissues were examined immunohistochemically. Subsets of memory T cells from isolated gingival cells were analyzed by flow cytometry using a cocktail of monoclonal antibodies (anti-CD69, anti-CD103, anti-CD45RA, anti-CCR7, anti-CD28, and anti-CD95). Intracellular cytokine staining of interleukin (IL)-17 and interferon (IFN)-γ expression on memory T cells in periodontitis tissues was also investigated.

Results: We found that healthy gingiva contains two memory T cell populations; a $CD69^-$ recirculating population and a $CD69^+$ gingiva-resident memory T cell population. $CD4^+$ T cells with transitional memory (T_{TM}) phenotype ($CD45RA^-CCR7^-CD28^+CD95^+$) constitute the major subset within these two populations. A significant increase in the proportion of $CD4^+CD69^+CD103^-$ memory T cells was observed in periodontitis tissues compared with healthy gingiva. $CD4^+$ memory T cells from periodontitis tissues produced either IL-17 or IFN-γ whereas $CD8^+$ memory T cells produced only IFN-γ.

Conclusions: Our findings suggest that recirculating and gingiva-resident memory T cells could represent an important part of the immune surveillance network in the connective tissue, maintaining periodontal homeostasis. Imbalance of subgingival bacterial communities could damage gingival barrier allowing bacterial antigens to get access to the deeper connective tissue where they activate memory T cells leading to deleterious inflammation; a hallmark of periodontitis.

KEYWORDS

gingiva, homeostasis, interleukin-17, memory, periodontitis, T-lymphocytes

Periodontitis involves a shift from a well-regulated immune homeostasis at a steady state to an environment that is driven by uncontrolled chronic immune responses leading to tissue damage and bone loss.[1] At steady state, the junctional epithelium is exposed to the dental plaque microbiome continually growing on the tooth surface.[2] Effective and balanced innate and adaptive immune responses at this site are crucial in maintaining immune homeostasis.[3] The first line

of defense depends on junctional epithelium integrity and its ability to control plaque bacteria growth via constant production of antimicrobial peptides.[2,4] The epithelium also secretes chemokines and cytokines including interleukin (IL)-8, IL-1β, and tumor necrosis factor (TNF)-α that upregulate adhesion molecules and recruit specific subsets of immune cells to the tissue site.[2] The other hallmark phenomenon involves the constant migration of polymorphonuclear neutrophils (PMNs) from blood vessels in the connective tissue and selectively migrate across the junctional epithelium into the gingival sulcus.[5] PMNs perform antibacterial functions including phagocytosis, antimicrobial peptide production, and neutrophil extracellular trap formation.[6] Antigen presenting cells including Langerhans cells, tissue dendritic cells, and macrophages have also been demonstrated to have immune regulatory function in maintaining homeostasis.[7–10] These well-controlled immune responses in healthy gingiva suppress the plaque bacteria and do not cause overt tissue pathology.[3] Unlike gut, gingiva does not have mucosal lymphoid tissues. However, a small number of B and T cells have been observed in healthy gingiva.[11] We recently described memory B cells residing in the connective tissue subjacent to the junctional epithelium in healthy gingiva suggesting that these cells may be involved in maintaining periodontal tissue wellbeing.[12]

Although the presence of T cells in healthy gingiva has been described, their memory phenotypic markers have not been clearly defined. Significant progress has been made during the past fifteen years in understanding the complexity of memory T cells, their trafficking and homing, transcriptional profile, and functions.[13] Different subsets of memory T cells include stem memory (T_{SCM}), central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}) and terminal effector memory (T_{TE}) T cells.[13]

T_{SCM} and T_{TM} cells have been recently identified. T_{SCM} cells have stem cell-like characteristics with their capacity to self-renew and also to generate more differentiated progeny from antigen stimulation.[14] It has been suggested that the T_{SCM} cells play an important role in immune-mediated disease.[15] T_{TM} cells were first identified in blood of healthy individuals and has been suggested that they are more differentiated compared with T_{CM} cells but not as fully differentiated as T_{EM} cells.[16,17]

A unique subset of memory T cells permanently resides in non-lymphoid tissue termed tissue-resident memory (T_{RM}) cells. CD69 is a key marker of T_{RM} while CD103 expression is found on a subset of $CD8^+$ and not $CD4^+$ T_{RM} cells.[18,19] C-type lectin CD69 inhibits sphingosine-1-phosphate receptor 1, leading to tissue retention,[20,21] whereas CD103 promotes cell adherence to E-cadherin expressed on tissue epithelial cells.[22] $CD69^+$ memory T cells have been commonly used to define tissue-resident population, whereas $CD69^-$ memory T cells represent a recirculating population.[4] $CD69^+$ T_{RM} cells consist of a core gene signature such as upregulation of chemokine receptor CXCR6 and also activation-induced molecules IL-2, IL-10, and PD-1 that can regulate proliferation.[18] Because of their strategic location in the mucosal tissues, $CD69^+$ T_{RM} cells have been shown to play a role in both protection and disease.[19,23–26]

A better understanding of memory T cells in periodontal health and disease could provide new insights into how these memory T cell subsets play a role in maintaining homeostasis and inducing destructive inflammatory responses. The purpose of this study was to characterize the composition of memory T cells in both healthy gingiva and periodontitis tissues. Our findings demonstrate that healthy gingiva-memory T cells comprise two populations; $CD69^-$ recirculating and $CD69^+$ gingiva-resident memory T cells. Effective immune response to keep homeostatic relationship with the dental plaque microbiome at the strategic area of the healthy gingival sulcus may partially rely on these memory T cells.

1 | MATERIALS AND METHODS

1.1 | Reagents

For flow cytometric analysis, monoclonal antibodies (mAbs) against the following proteins were used: CD3 (SK7),* CD4 (RPA-T4),* CD8 (RPA-T8),* CD 28 (CD28.2),* CD95 (DX2),* CD45RA (5H9),* CCR7 (150503),* CD69 (FN50),* CD103 (Ber-ACT8),* IFN-γ (B27),* and IL-17A (eBio64CAP17).† For immunohistochemistry analysis, mAbs against the following proteins were used: CD3 (A0452),‡ CD4 (EPR6855) and CD103 (EPR4166(2)),§ and CD8 (1A5).¶

1.2 | Ethics statement

Ethical approval was obtained from the Ethics Committee Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2013–016). Written informed consent of all participants was obtained prior to inclusion in the study.

1.3 | Patients and gingival tissue specimens

Forty-one Thai participants at the Periodontal Clinic and Oral Maxillofacial Surgery Clinic, Faculty of Dentistry, Chulalongkorn University were recruited between July 2015 and July 2017 (see supplementary Table 1 in online *Journal of Periodontology*). All participants were in good general health, non-smokers, non-diabetes, and had not taken antimicrobial or anti-inflammatory drugs within the past 3

* BD Biosciences, San Jose, CA.

† eBioscience, San Diego, CA.

‡ DAKO, Glostrup, Denmark.

§ Abcams, Cambridge, U.K.

¶ Leica Biosystems, Newcastle, U.K.

months. Twenty-four participants (14 males and 10 females, aged 30 to 77 years; mean age: 53.25 ± 2.31 years) were classified as chronic severe periodontitis according to the International Workshop (1999)[27] (see supplementary Table 1 in online *Journal of Periodontology*). Periodontitis tissue specimens were collected from sites of extracted teeth with hopeless prognosis.[28] Periodontitis sites showed bleeding on probing, probing depth > 6 mm, severe clinical attachment loss, and bone loss > 60% of the root, thus indicating inadequate attachment to maintain the tooth (see supplementary Table 2 in online *Journal of Periodontology*). Seventeen participants (seven males and 10 females, aged 17 to 66 years; mean age: 43.41 ± 4.71 years) had healthy gingiva with no sign of gingival inflammation (no bleeding on probing), probing depth < 4 mm and no clinical attachment loss (see supplementary Table 2 in online *Journal of Periodontology*). Healthy tissue specimens were collected during crown-lengthening procedure for prosthetic or orthodontic reasons. From each participant, a gingival tissue specimen was obtained from one selected site according to and prepared for either flow cytometric or immunohistochemical analysis. Each tissue specimen was approximately 0.2 to 0.4 cm x 0.2 cm in size.

1.4 | Immunohistochemistry

Gingival tissues from healthy (n = 7) and periodontitis patients (n = 11), one sample per patient, were fixed in 10% buffered formalin for a maximum of 24 hours and subsequently embedded in paraffin. Microtome serial paraffin sections (4 μm in thickness) were cut from the central part of each specimen in a plane parallel to the long axis of the teeth and oriented so that the pocket epithelium or the sulcular epithelium, oral epithelium, and connective tissues were present in the same section. The sections were mounted on a glass slide. Sections were deparaffinized. To inhibit endogenous peroxidase, the sections were incubated in 0.3% hydrogen peroxide solution for 20 minutes and heated in 1 mM EDTA pH 8.0 at 95°C for 20 minutes for antigen retrieval.

To identify $CD3^+$, $CD4^+$, and $CD8^+$ cells, staining was performed on the sections via polymer/HRP and DAB^+chromogen system.[‡] The samples were stained with the designated primary antibodies followed by Polymer/HRP, and then counterstained with hematoxylin.

1.5 | Gingival cell preparation

Gingival tissues were washed three to four times in Roswell Park Memorial Institute (RPMI) 1640 medium and cut into small fragments (1 to 2 mm^3). The fragments were incubated in medium that contained 2 mg/mL collagenase type I.* After 90 minutes incubation (37°C), residual tissue fragments were disaggregated by gentle flushing, until single-cell suspensions were obtained. These cells were filtered through a 70-μm mesh size filter and then used for flow cytometry analysis and cytokine study.

1.6 | Flow cytometric analysis of memory T cell subsets

Cells from healthy gingiva (n = 9) and periodontitis tissues (n = 4) were stained with mAbs against CD3, CD4, CD8, CD28, CD95, CD45RA, and CCR7. Staining with the antibody cocktail containing antibodies specific to CD45RA, CCR7, CD28, and CD95, enabled us to simultaneously identify naïve and five subsets of memory T cells.[13] T cells expressing $CD45RA^+CCR7^+CD28^+CD95^-$ were defined as naïve T cells. Phenotypic markers of memory T cell subsets were characterized as follows:1) $CD45RA^+CCR7^+CD28^+CD95^+$ cells were T_{SCM} cells, 2) $CD45RA^-CCR7^+CD28^+CD95^+$ cells were T_{CM} cells, 3) $CD45RA^-CCR7^-CD28^+CD95^+$ cells were T_{TM} cells, 4) $CD45RA^-CCR7^-CD28^-CD95^+$ cells were T_{EM} cells, and 5) $CD45RA^+CCR7^-CD28^-CD95^+$ cells were T_{TE} cells.

To identify T_{RM} cell markers; CD69 and CD103 cells were stained with mAbs against CD3, CD4, CD8, CD69, and CD103. $CD69^-$ memory T cells were characterized as recirculating memory T cells, while $CD69^+$ memory T cells were characterized as gingiva-resident memory T cells. Flow cytometry analysis was performed using 12-color flow cytometry.[†,‡]

1.7 | Intracellular staining analysis of IL-17 and IFN-γ

Single-cell suspensions obtained from gingival tissues were stimulated with Staphylococcal enterotoxin (SEB)[§] (4 μg/mL) and gingival cells cultured in medium served as negative control. After 2 hours of stimulation, Golgiplug* was added to inhibit cytokine secretion and the cell cultures were further incubated overnight. The cells were washed and stained with a panel of antibodies including anti-CD3, anti-CD4, anti-CD8, and anti-CD103 mAbs. The stained cells were fixed/permeabilized* and intracellular cytokines were stained with mAbs against IL-17 and IFN-γ. Finally, stained cells were analyzed by a 12-color flow cytometer.[‡‡]

1.8 | Statistical analysis

The data were analyzed using a statistical analysis program.*** Results were shown as mean ± SEM. Comparisons of one variable between two groups were analyzed

* Gibco, Grand Island, NY.

† FACSCelesta, BD Biosciences, San Jose, CA.

‡ Sigma-Aldrich, St. Louis, MO.

§ SPSS 22.0 for Windows, SPSS, Chicago, IL.

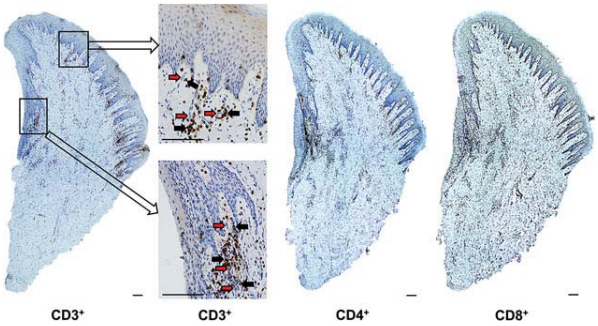

FIGURE 1 Anatomical compartmental location of T cells in healthy gingiva. Representative immunostaining of $CD3^+$, $CD4^+$, and $CD8^+$ T cells from seven different healthy individuals. Black arrows indicate $CD3^+$ T cells. Red arrows indicate capillary in the connective tissue. Scale bars = 100 μm

using the Mann-Whitney *U* test. Comparisons between two variables in one group were analyzed using the Wilcoxon signed-rank test. Two-tailed *P* values < 0.05 were considered statistically significant.

2 | RESULTS

2.1 | Human memory T cells in healthy gingiva

Immunohistochemical staining was used to investigate the presence and anatomical location of T cells in healthy gingiva. Figure 1 showed that $CD3^+$ T cells formed clusters close to capillary opening, scattered throughout connective tissue. Some were detected in the epithelial layer. Dense $CD3^+$ T cell clusters were consistently observed at the bottom of the healthy gingival sulcus adjacent to the junctional epithelium. $CD4^+$ and $CD8^+$ T cells were also detected in the same locations (see supplementary Figure 1 in online *Journal of Periodontology*).

Most of the T cells isolated from healthy gingiva were memory T cells (see supplementary Figure 2A in online *Journal of Periodontology*). The proportion of naïve T cells was negligible (< 0.5%) suggesting no blood contamination (data not shown). The percentage of $CD4^+$ memory T cells in healthy gingiva was consistently higher compared with $CD8^+$ memory T cells (61.52% ± 1.47% versus 38.48% ± 1.47%, $P < 0.001$, (see supplementary Figure 2B in online *Journal of Periodontology*). We next analyzed the expression of CD69 and CD103, surface markers commonly used to identify T_{RM} cells. T cells expressing CD69 were defined as gingiva-resident memory T cells whereas those not expressing CD69 were defined as recirculating memory T cells. Healthy gingiva contained both $CD69^-$ recirculating memory T cells (56.26% ± 4.31%) and $CD69^+$ gingiva-resident memory T cells (43.74% ± 4.31%; $P = 0.203$) (Figure 2A). Most of $CD69^-$ and $CD69^+$ memory T cell populations were $CD4^+$ cells and were CD103 negative ($CD4^+CD69^-CD103^-$ T cell frequency = 34.21% ± 3.17%; $CD4^+CD69^+CD103^-$ T cell frequency = 26.68% ± 2.96%). Only a small proportion of memory T cells expressed CD103, which was mostly detected on $CD8^+$ T cells.

We next characterized the composition of the memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in the recirculating and gingiva-resident memory T cell populations. Figure 2B showed that $CD45RA^-CCR7^-CD28^+CD95^+$ T_{TM} cells (73.17% ± 3.71%) and $CD45RA^-$ $CCR7^+CD28^+CD95^+$ T_{CM} cells (22.17% ± 3.13%) composed the majority of $CD4^+CD69^-CD103^-$ recirculating memory T cells. The percentages of T_{SCM}, T_{EM}, and T_{TE} cells were negligible (0.1% to 2%). Within the $CD8^+CD69^-$ $CD103^-$ recirculating memory T cells, the T_{TM} cells comprised 48.42% ± 4.43%, followed by $CD45RA^-CCR7^-CD28^-CD95^+$ T_{EM} (29.10% ± 4.53%), $CD45RA^+CCR7^-CD28^-CD95^+$ T_{TE} (11.53% ± 2.43%), $CD45RA^+CCR7^+CD28^+CD95^+$ T_{SCM} (5.73% ± 0.99%), and T_{CM} cells (5.16% ± 0.62%). The proportion of $CD69^-CD103^+$ recirculating population was minimal and their memory T cell subset compositions was similar to those in the $CD69^-CD103^-$ recirculating population (Figure 2C).

The composition of the memory T cell subsets of the $CD69^+$ gingiva-resident memory T cells was evaluated. T_{TM} and T_{CM} cells made up 77.55% ± 1.87% and 19.72% ± 2.03%, respectively, of the $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells. The percentages of T_{SCM}, T_{EM}, and T_{TE} cells were negligible (0.14% to 2.15%). Analysis of the $CD8^+CD69^+CD103^-$ gingiva-resident memory T cells indicated that the T_{TM} cells constituted 60.73% ± 3.69%, followed by T_{TE} (22.82% ± 3.71%), T_{EM} (6.21% ± 1.23%), T_{CM} (5.39% ± 1.44%) and T_{SCM} cells (4.85% ± 1.01%) (Figure 2D).

Only a small population of the $CD69^+$ gingiva-resident T cells expressed CD103. In the $CD4^+CD69^+CD103^+$ gingiva-resident memory T cells, most were T_{TM} cells (83.14%), followed by T_{CM} (13.39% ± 2.00%), T_{EM} (3.169% ± 2.15%), and T_{SCM} cells (0.05% ± 0.04%). Among the $CD8^+CD69^+CD103^+$ gingiva-resident memory T cells, the majority were T_{TM} cells (68.01% ± 4.18%), followed by T_{EM} (15.60% ± 3.41%), T_{TE} (10.17% ± 2.32%), T_{CM} (5.53% ± 1.2%), and T_{SCM} cells (0.69% ± 0.27%) (Figure 2E). Q10 Q11

Taken together, the data suggest that $CD4^+$ recirculating memory T cells and $CD4^+$ gingiva-resident memory T cells with T_{TM} cell phenotype represent the majority of T cell subsets in healthy gingiva.

2.2 | Increased accumulation of memory T cells in periodontitis tissues

Immunohistochemical staining of periodontitis tissues showed an increase of $CD3^+$ T cells at the base of the periodontal pocket area and scattered throughout the connective

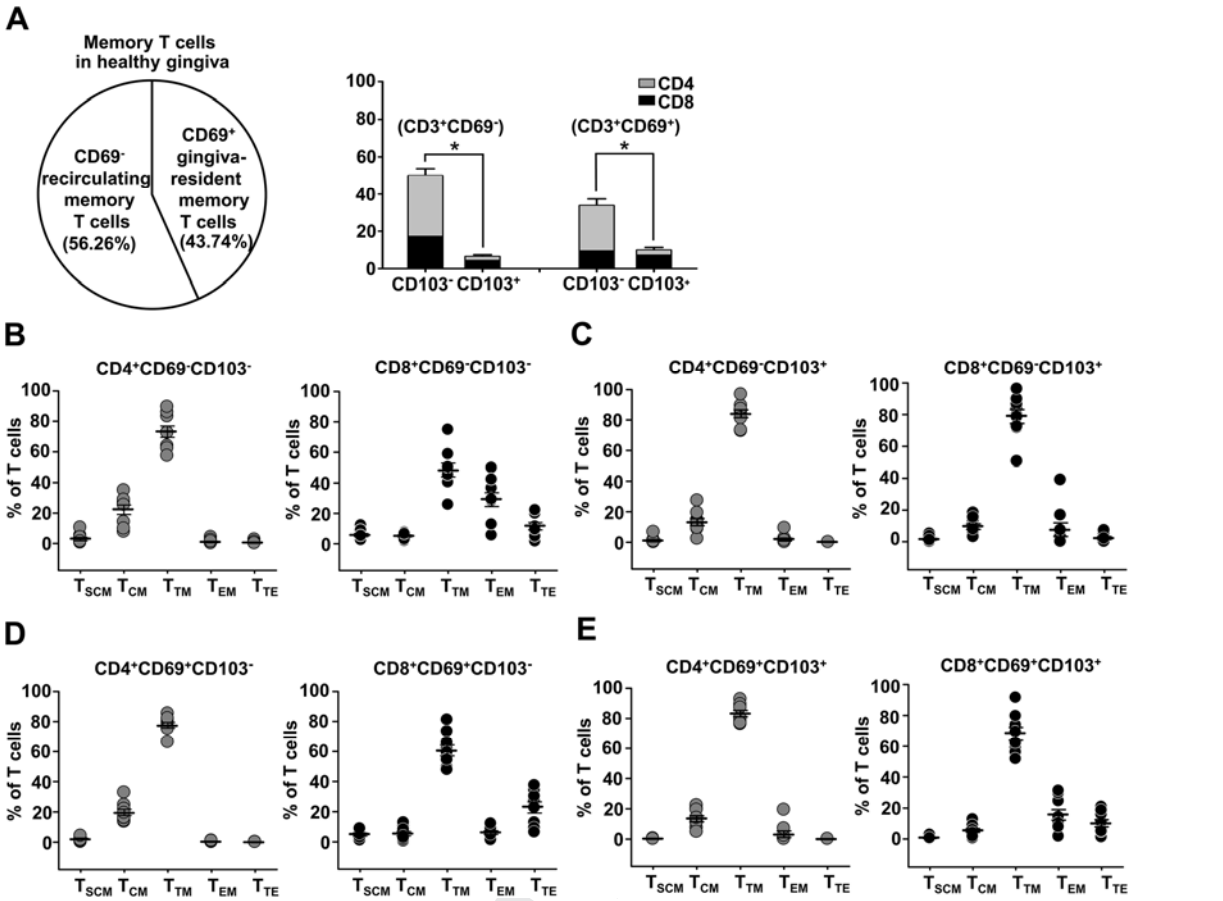

FIGURE 2 Composition of memory T cell subsets in healthy gingiva. (A) Percentage distribution of $CD69^-$ recirculating memory T cells and $CD69^+$ gingiva-resident memory T cells. Means ± SEM of n = 9 are shown. The composition of memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in (B) $CD4^+CD69^-CD103^-$ and $CD8^+CD69^-CD103^-$, (C) $CD4^+CD69^-CD103^+$ and $CD8^+CD69^-CD103^+$, (D) $CD4^+CD69^+CD103^-$ and $CD8^+CD69^+CD103^-$, (E) $CD4^+CD69^+CD103^+$ and $CD8^+CD69^+CD103^+$. Each data point represents the value of an individual sample and means ± SEM are shown (n = 9)

tissue, especially apically toward the advancing front of the lesion. Increased $CD3^+$ T cells in the epithelial layer were not observed (Figure 3). Numerous $CD4^+$ and $CD8^+$ T cells were also detected in the connective tissue (see supplementary Figure 3 in online *Journal of Periodontology*). Similar to healthy gingiva, most of the T cells isolated from periodontitis tissues were memory T cells (see supplementary Figure 2C in online *Journal of Periodontology*) and a higher proportion of $CD4^+$ compared with $CD8^+$ (65.03% ± 5.24% versus 34.97% ± 5.24%; $P < 0.05$) memory T cells was observed (see supplementary Figure 2D in online *Journal of Periodontology*).

Interestingly, the proportion of $CD4^+CD69^-CD103^-$ recirculating memory T cells was significantly lower in periodontitis tissues compared with healthy gingiva (32.40% ± 3.03% versus 22.33% ± 2.54%; $P = 0.037$). In contrast we observed an increased proportion of $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells compared with healthy gingiva (36.85% ± 3.22% versus 24.11% ± 2.70%; $P = 0.017$) (Figure 4A and Figure 2A).

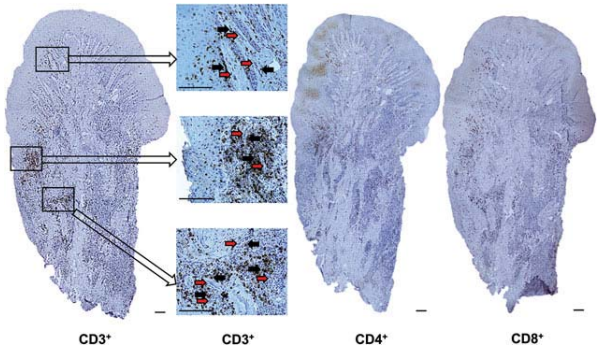

FIGURE 3 Anatomical compartmental location of T cells in periodontitis tissue. Representative immunostaining of $CD3^+$, $CD4^+$, and $CD8^+$ T cells from 11 different periodontitis patients. Black arrows indicate $CD3^+$ T cells. Red arrows indicate capillary in the connective tissue. Scale bars = 100 μm

The composition of the memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in periodontitis tissues were similar to those in healthy gingiva. $CD4^+$ and $CD8^+$ T_{TM}

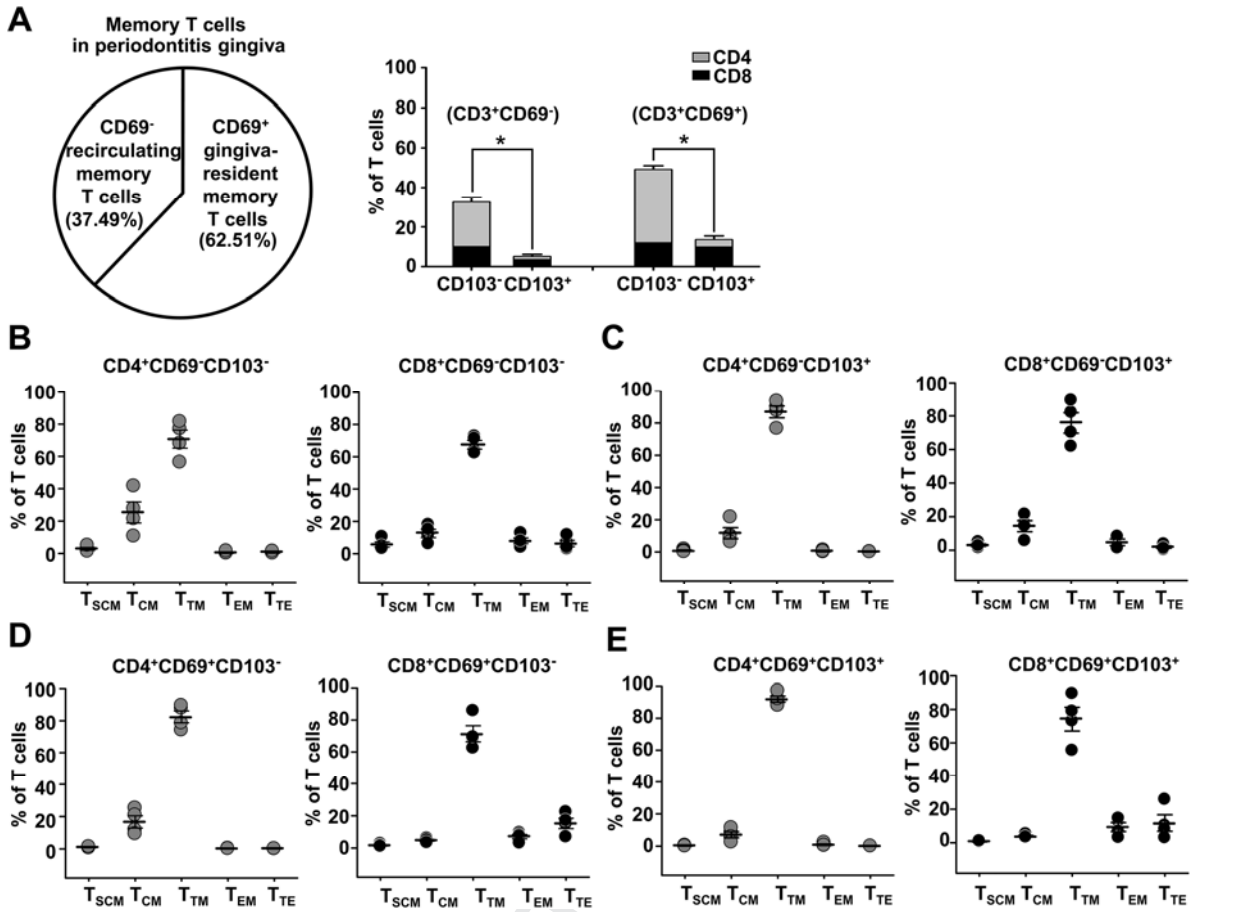

FIGURE 4 Composition of memory T cell subsets in periodontitis tissue. (A) Percentage distribution of $CD69^-$ recirculating memory T cells and $CD69^+$ gingiva-resident memory T cells. Means ± SEM of n = 4 are shown. The composition of memory T cell subsets; T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells in (B) $CD4^+CD69^-CD103^-$ and $CD8^+CD69^-CD103^-$, (C) $CD4^+CD69^-CD103^+$ and $CD8^+CD69^-CD103^+$, (D) $CD4^+CD69^+CD103^-$ and $CD8^+CD69^+CD103^-$, (E) $CD4^+CD69^+CD103^+$ and $CD8^+CD69^+CD103^+$. Each data point represents the value of an individual sample and means ± SEM are shown (n = 4)

cells (67.40% to 91.91%) constituted the major subset in both recirculating and gingiva-resident memory T cell populations (Figures 4B through 4E).

Large numbers of infiltrated T cells together with the increased proportion of $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells in periodontitis tissues may suggest the possible role in pathogenesis of this memory T cell subset.

2.3 | IL-17 and IFN-γ responses from memory T cells isolated from periodontitis tissues

The small number of T cells that could be isolated from healthy gingiva limited the study of IL-17 and IFN-γ responses. Cytokine responses from T cells derived from periodontitis tissues are shown in Figure 5A. Memory T cells produced both IL-17 and IFN-γ. $CD4^+$ memory T cells predominant produced IL-17 alone (frequency = 1.88% ± 0.52%) and IFN-γ alone (frequency = 3.42% ± 1.82%). The frequency of $CD4^+$ T cells that produced IL-17 plus IFN-γ was negligible. Unlike $CD4^+$ memory T cells, $CD8^+$ memory T cells mostly produced IFN-γ alone (frequency = 8.51% ± 1.41%).

Measurement of IL-17 and IFN-γ production in recirculating versus gingiva-resident memory T cells based on CD69 expression was not possible because in vitro stimulation with SEB leads to CD69 expression on most T cells.[29] We next compared the cytokine response between the memory T cell $CD103^+$ and $CD103^-$ subsets. We found that the frequency of $CD4^+CD103^+$ memory T cells that produced IFN-γ was 4.4-fold higher than that of $CD4^+CD103^-$ memory T cells however, the differences did not reach statistical significance (12.87% ± 4.82% versus 2.92% ± 1.55%; $P = 0.06$) (Figure 5B). The frequency of IL-17 producing cells between $CD4^+CD103^+$ and $CD4^+CD103^-$ memory T cells was similar (1.49% ± 0.25% versus 1.92% ± 0.51%). A significant difference in the frequency of IFN-γ producing cells between $CD8^+CD103^+$ and $CD8^+CD\ 103^-$ memory T cells was observed (11.40% ± 1.02% versus 7.56% ± 1.07%; $P = 0.029$).

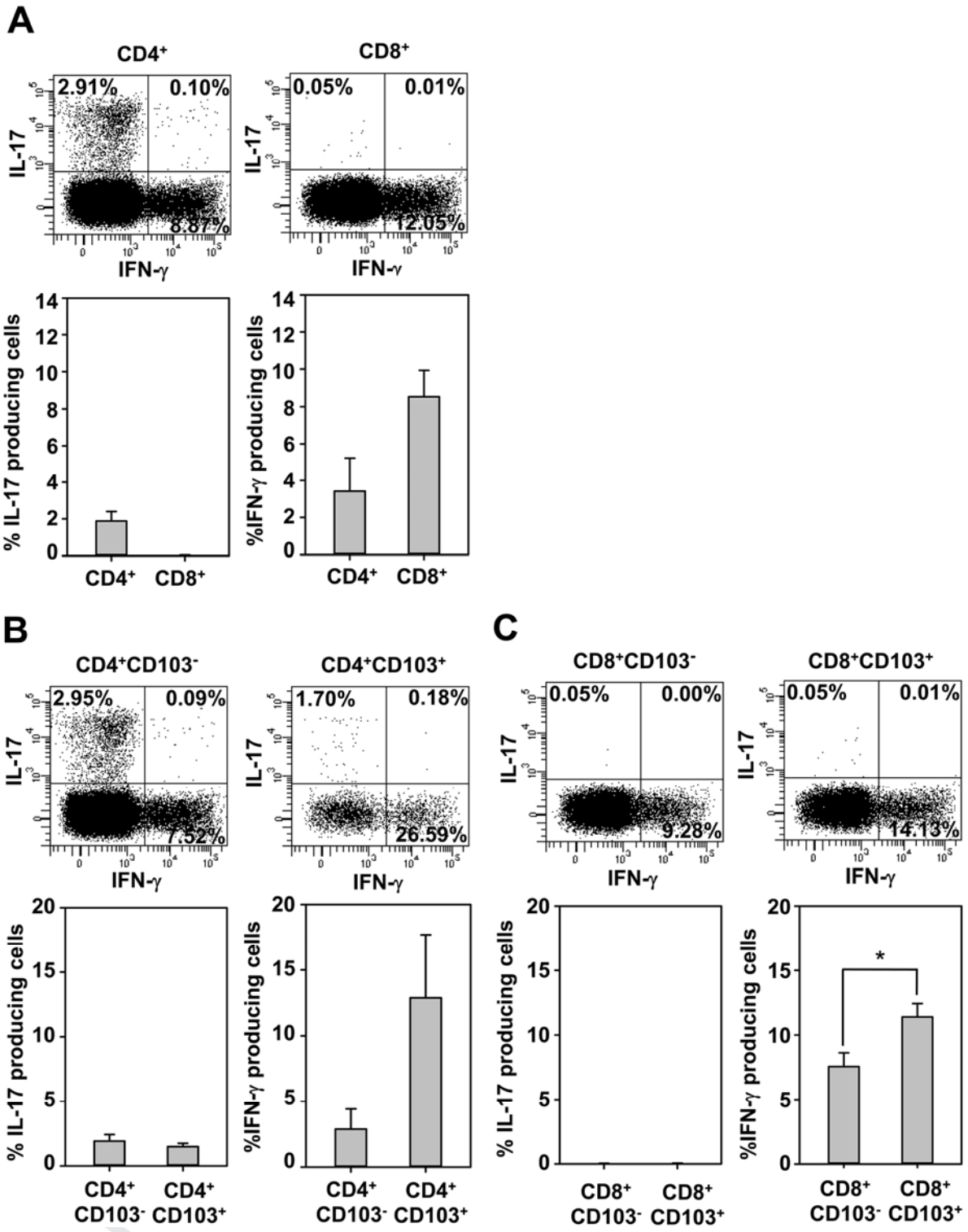

FIGURE 5 Expression of IL-17 and IFN-γ of $CD4^+$ and $CD8^+$ memory T cells isolated from periodontitis tissue. (A) Representative of flow cytometry plot of $CD4^+$ and $CD8^+$ memory T cells expressing IL-17 and IFN-γ and frequencies of IL-17 and IFN-γ production from $CD4^+$ and $CD8^+$ memory T cells. Mean ± SEM of n = 4 are shown. (B) Representative of flow cytometry plot of $CD4^+CD103^-$ and $CD4^+CD103^+$ memory T cells expressing IL-17 and IFN-γ and frequencies of IL-17 and IFN-γ production from $CD4^+CD103^-$ and $CD4^+CD103^+$ memory T cells. Mean ± SEM of n = 4 are shown. (C) Representative of flow cytometry plot of $CD8^+CD103^-$ and $CD8^+CD103^+$ memory T cells expressing IL-17 and IFN-γ and frequencies of IL-17 and IFN-γ production from $CD8^+CD103^-$ and $CD8^+CD103^+$ memory T cells. Mean ± SEM of n = 4 are shown

IL-17 has been shown to be an important mediator in causing bone loss.[30] Our findings that $CD4^+$memory T cells are the major source of IL-17 suggest that these cells are potentially involved in pathogenesis of periodontitis.

3 | DISCUSSION

The role of memory T cells, especially T_{RM} cells in health and disease has received a lot of attention lately.[19,23–26] There have been a few reports on T cells in healthy gingiva.[31–33] Using immunohistochemical staining and flow cytometry, we showed that, like other human mucosal tissues, most T cells isolated from healthy gingiva had a memory phenotype. The two major populations of these memory T cells are recirculating and gingiva-resident memory T cells, characterized by the expression of CD69, a well-known T_{RM} retention marker. Gingival memory T cells formed clusters scattered in the connective tissue, preferentially subjacent to the junctional epithelium, a strategic area vulnerable to subgingival plaque microbiome. Our findings highlight the possible role of memory T cells in the healthy gingiva immune surveillance network.

$CD4^+$ memory T cells with a T_{TM} cell phenotype were the major subset in both $CD69^-$ recirculating and $CD69^+$ gingiva-resident memory T cells. $CD8^+$ memory T cells in healthy gingiva represented a minority subset and had T_{TM} cell phenotype. These results were different from other studies showing that tissue T cells had a T_{EM} cell phenotype.[4,34] Resident T cells with T_{EM} cell phenotype were also reported in healthy gingiva.[33] These discrepancies may result from using different cell surface markers for memory T cell phenotype identification. While most studies used $CD45RA^-CCR7^-$ to define the T_{EM} cell phenotype, we included two additional markers, anti-CD28 and anti-CD95 mAbs in the antibody cocktail, allowing subset differentiation between T_{TM} cells ($CD45RA^-CCR7^-CD28^+CD95^+$) and T_{EM} cells ($CD45RA^-CCR7^-CD28^-CD95+$). T_{TM} cells were first identified in blood of healthy individuals and has been suggested that they are more differentiated compared with T_{CM} cells but not as fully differentiated as T_{EM} cells.[16,17] The role of T_{TM} cells in protection and disease has been poorly described. Recent data suggest that $CD4^+$ T_{TM} cells could serve as a reservoir of latent HIV infection.[35] Further study is needed to better understand the role of $CD4^+$ T_{TM} cells in periodontal health and disease.

Immunohistochemical staining of periodontitis tissues revealed the increase of memory T cell clusters at the base of the periodontal pocket and scattered throughout the gingiva, especially apically toward the advancing front of the lesion. Similar to healthy gingiva, $CD4^+$ T_{TM} cells also comprised the major subset in recirculating and gingiva-resident memory T cell populations. Interestingly, we detected a significant increase in the proportion of $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells in periodontitis tissues compared with healthy gingiva. This increase could result from a selective expansion of $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells that pre-exist in healthy gingiva. Alternatively, the expansion of these cells may result from the pre-existing $CD4^+CD69^+CD103^-$ memory T cells combined with some of the recirculating $CD4^+CD69^-$ $CD103^-$ memory T cells that become activated and express CD69 during the course of disease (CD69 expression in this case, represents an early T cell activation marker). One could speculate that the increase of $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells could play a role in pathogenesis of periodontitis.

$CD4^+$ memory T cells isolated from periodontitis tissues produced either IL-17 or IFN-γ while $CD8^+$ memory T cells produced only IFN-γ. These findings agree with recent observations suggesting that the major source of IL-17 in periodontitis is $CD4^+$ memory T cells.[33] $CD103^+$ memory T cells express higher levels of cytokines compared with $CD103^-$ memory T cells.[36] In this study, we did not observe the differences in the magnitude of IL-17 and IFN-γ responses between $CD4^+CD103^+$ and $CD4^+CD103^-$ memory T cells. However, $CD8^+CD103^+$ memory T cells showed a significant higher IFN-γ response compared with $CD8^+CD103^-$ memory T cells.

Because of technical limitations, the number of T cells isolated from healthy gingiva was too low which limited cytokine investigation. However, data from single healthy tissue samples showed that memory T cells produced both IL-17 and IFN-γ, similar to those from periodontitis tissues (see supplementary Figure 4 in online *Journal of Periodontology*). Our observations confirm recent studies describing that $CD4^+$ memory T cells from healthy gingiva produced IL-17 and IFN-γ.[33,37]

The role of IL-17 and IFN-γ in pathogenesis of periodontitis has been investigated. IL-17 has been detected in periodontitis and is responsible for tissue damage and inflammatory bone loss.[30,38] High expression of IFN-γ has been consistently detected in human periodontitis tissues.[39,40] In mice that lack an IFN-γ response, the animals develop less gingival tissue damage and bone loss following *Aggregatibacter actinomycetemcomitans* infection.[41]

Several evidences also point out tissue-protective and immune regulatory functions of IL-17 and IFN-γ in maintaining immune homeostasis. IL-17 stimulates antimicrobial peptide production by gut epithelial cells and maintain epithelial cell tight junctions at steady state.[42] IL-17 also promotes PMN migration across epithelium via IL-17- mediated IL-8 production.[43] The role of IFN-γ in maintaining immune homeostasis is less well described. A recent study suggests that IFN-γ regulates homeostasis in healthy tissue by upregulation of suppressor-of-cytokine-2 (SOCS2) protein

expressed by tissue phagocytes.[44] Moreover, we previously described that IFN-γ induces indoleamine 2, 3-dioxygenase production from gingival fibroblast cells.[45] This immune suppressing enzyme has been suggested to be a crucial mediator in maintaining gut homeostasis.[46] The ability of IFN-γ to upregulate adhesion molecule VCAM-1 on endothelial cells[47] and ICAM-1 on gingival fibroblasts[45] may be responsible for the recruitment and retention of recirculating memory T cells observed in healthy gingiva. Collectively, these data suggest that IL-17 and IFN-γ responses, especially at low levels may have a beneficial role in supporting gingival tissue homeostasis and controlling tissue inflammation.

It should be pointed out that the current study was conducted using small sample size, limiting conclusions to some degree. Another limitation is that we did not determine the frequency of regulatory T (T_{REG}) cells in healthy gingiva and periodontitis tissues. The function of T_{REG} cells has been demonstrated to counterbalance inflammatory responses.[48] A better understanding of how T_{REG} cells may be involved in immune homeostasis and inflammation in human gingiva is required.

4 | CONCLUSIONS

Classic notions regarding the crucial role of innate cells (junctional epithelium and PMN) in maintaining gingiva health have been long held. Our findings reveal that memory T cells may be involved in the healthy gingiva immune surveillance network and together with the innate arm of immune defense could maintain a homeostatic relationship with subgingival plaque microbiome. Microbial imbalance could damage the epithelial barrier allowing large amounts of bacteria and their antigens to gain access to the connective tissue. These antigens could provoke uncontrolled T cell activation leading to the increase of $CD4^+CD69^+CD103^-$ gingiva-resident memory T cells. Cytokine production from this subset of memory T cells may be responsible for tissue damage and bone loss in periodontitis. Further investigation to characterize memory T cell antigen specificity, the role of subgingival plaque bacteria, and specific tissue signals that promote the localization of recirculating and gingiva-resident memory T cells will provide additional insights into how the gingival immune response operates in health and disease.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Human Memory B Cells in Healthy Gingiva, Gingivitis, and Periodontitis

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The presence of inflammatory infiltrates with B cells, specifically plasma cells, is the hallmark of periodontitis lesions. The composition of these infiltrates in various stages of homeostasis and disease development is not well documented. Human tissue biopsies from sites with gingival health (n = 29), gingivitis (n = 8), and periodontitis (n = 21) as well as gingival tissue after treated periodontitis (n = 6) were obtained and analyzed for their composition of B cell subsets. Ag specificity, Ig secretion, and expression of receptor activator of NF-κB ligand and granzyme B were performed. Although most of the B cell subsets in healthy gingiva and gingivitis tissues were $CD19^+CD27^+CD38^-$ memory B cells, the major B cell component in periodontitis was $CD19^+CD27^+CD38^+CD138^+HLA\text{-}DR^{low}$ plasma cells, not plasmablasts. Plasma cell aggregates were observed at the base of the periodontal pocket and scattered throughout the gingiva, especially apically toward the advancing front of the lesion. High expression of CXCL12, a proliferation-inducing ligand, B cell–activating factor, IL-10, IL-6, and IL-21 molecules involved in local B cell responses was detected in both gingivitis and periodontitis tissues. Periodontitis tissue plasma cells mainly secreted IgG specific to periodontal pathogens and also expressed receptor activator of NF-κB ligand, a bone resorption cytokine. Memory B cells resided in the connective tissue subjacent to the junctional epithelium in healthy gingiva. This suggested a role of memory B cells in maintaining periodontal homeostasis. ***The Journal of Immunology*****, 2016, 197: 715–725.**

The Global Burden of Disease Study (2010) ranked severe periodontitis as the sixth-most prevalent disease in the world, affecting 11.2% worldwide (1).

Chronic periodontitis is an advanced form of periodontal diseases and represents a chronic inflammation of tooth-supporting structures. It is characterized by gingival inflammation (redness, swelling, and bleeding) and loss of connective tissue attachment to the tooth that may result in resorption of the alveolar bone. Thus, an advanced stage of periodontitis may eventually lead to tooth loss. The precursor stage of periodontitis is limited to the marginal gingival region and is called gingivitis. It is characterized by gingival inflammation without the loss of attachment and alveolar bone.

In the 1960s to 70s, the etiologic role of dental plaque in periodontal diseases was firmly established. In the same period, an intense effort was made to understand periodontal immunopathogenesis, which involves the interplay between the dental plaque biofilm and the host response.

Classical human experimental gingivitis (2, 3) and experimental periodontitis (4) studies in animal models outlined changes in microbial plaque and tissue immunopathology during the transition from periodontal health to gingivitis and, later on, to periodontitis. Increases in the Gram-positive bacterial species in supragingival plaque biofilms such as *Streptococcus* and *Actinomyces* species were observed concomitant with the development of a T cell–dominated gingivitis infiltrate. The presence of Gram-negative bacterial species in subgingival plaque biofilms such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tanerella forsythia*, and *Treponema denticola* was later associated with the development of plasma cell–dominated periodontitis lesions (5, 6).

The T cell response to the presence of periodontal pathogens is considered to play a key role in the regulation of periodontal pathogenesis. It has been speculated that a Th2 response was associated with periodontitis, whereas Th1 responses are associated with gingivitis (7). However, such a hypothesis remains controversial. More recently, other Th subsets, including regulatory Th cells and Th17 cells, have been demonstrated in periodontal tissues, of which an inverse relationship between these two cell types could be established (8). Whereas Th17 cells promote periodontal inflammation and tissue destruction (9), T regulatory cells are implicated in guard against periodontitis progression (10).

To date, emphasis has been placed on the T cell response in controlling local immunity and causing chronic periodontal tissue destruction. However, the role of the B cell response in periodontal

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Abbreviations used in this article: APRIL, a proliferation-inducing ligand; ASC, Ab-secreting cell; BAFF, B cell–activating factor; DAB, diaminobenzidine; DPBS, Dulbecco PBS; MFI, mean fluorescence intensity; PBST, PBS with 0.05% Tween 20; PNAd, peripheral node addressin; RANKL, receptor activator of NF-κB ligand; SFC, spot-forming cell.

homeostasis or disease development has not been thoroughly studied. Since 1965, it has been known that Ig-producing plasma cells predominated the periodontitis lesions (11). B cells and plasma cells outnumbered T cells, when gingivitis progressed to periodontitis (5). However, detailed profiles of B cell subsets, including naive B cells, memory B cells, and Ab-secreting cells (ASCs), had not yet been investigated systematically in periodontal tissues of various healthy or diseased conditions.

Human B cell subsets may now be identified phenotypically based on the expression of different surface markers, such as CD19, CD20, CD27, CD38, and CD138 (12, 13). A pan B cell marker, CD19, is expressed in all B cell subsets, whereas CD20 is downregulated, when memory B cells differentiate into ASCs (14). CD27, a member of the TNFR family, which induces differentiation and promotes survival (15), is expressed on the majority of memory B cells (16). CD38 catalyzes the formation of cyclic ADP-ribose and NADP and regulates Ca^{+} signaling in lymphoid cells (17). Both CD27 and CD38 are highly expressed on ASCs. CD138, a heparin sulfate proteoglycan, also known as syndecan-1, is generally used as a plasma cell marker (18).

The purpose of this study was to identify different B cell subsets in clinically healthy and diseased periodontal tissues. Moreover, the morphological compartmental localization of the cells and hence their possible role in disease development were to be explored.

Materials and Methods

Reagents

Medium for gingival cell culture. The gingival cells were cultured in complete medium consisting of RPMI 1640 supplemented with nonessential amino acids, 2 mM *l*-glutamine, 1 mM sodium pyruvate, 100 μg/ml penicillin, 100 μg/ml streptomycin (Life Technologies Laboratories, Grand Island, NY), and 10 μg/ml 2-ME, supplemented with 10% FCS (Hyclone).

Abs. Abs were used against the following proteins for flow cytometry: CD3 (SK7), CD19 (4G7), CD27 (M-T271), CD38 (HIT2), CD138 (MI15), HLA-DR (L243), and granzyme B (GB11) from BD Biosciences; receptor activator of NF-κB ligand (RANKL; MIH24) from BioLegend. Abs were used against the following proteins for immunohistochemistry analysis: CD20 (L26) and CD27 (137B4) from Abcam; CD138 (MI15) and peripheral node addressin (PNAd; MECA-79) from BioLegend; follicular dendritic cells (CNA.2) from eBioscience; ICAM-1 (23G12) from Thermo Scientific; and CD62L (B-8) from Santa Cruz Biotechnology.

Ethics statement

The ethical approval was obtained from the Ethics Committee of Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2013-016). Written informed consent of all participating subjects was obtained prior to inclusion in the study.

Periodontal tissue specimens and peripheral blood collection

Periodontal tissues and heparinized peripheral blood samples were obtained from patients with severe chronic periodontitis and gingivitis and subjects with clinically healthy gingiva. These specimens were collected from patients at Periodontal Clinic and Oral Maxillofacial Surgery Clinic, Faculty of Dentistry, Chulalongkorn University (Bangkok, Thailand).

Each patient had at least 16 remaining teeth with no history of periodontal treatment for the past 6 mo. Subjects with clinically healthy gingiva showed no sign of gingival inflammation (no bleeding on probing, probing depth <4 mm) and no clinical attachment loss or bone loss. Healthy gingival tissue specimens were collected during crown-lengthening procedure for prosthetic or orthodontic reasons. Gingivitis tissue specimens were collected from inflamed sites (bleeding on probing, but no clinical attachment or bone loss) of extracted tooth due to tooth malposition, crowding, or pericoronitis. Periodontitis tissue specimens were collected from sites of extracted teeth irrational to treat (bleeding on probing, probing pocket depth >6 mm, and bone loss >60% of the root). After periodontal treatment of scaling and root planing in four subjects with severe chronic periodontitis, treated tissue specimens (negligible sign of marginal gingival inflammation, but advanced clinical attachment loss and bone loss >60% of the root) were collected from teeth extracted as being judged irrational to treat. All subjects were in good general health, and none of them had taken antimicrobial or anti-inflammatory drugs within the previous 3 mo.

The excised tissues were immediately placed in sterile tubes that contain RPMI 1640 medium. Three milliliters of peripheral blood was collected. The samples were transferred to the laboratory within a few hours for the study.

Gingival cell preparation

The method for obtaining single-cell suspensions from gingival tissues was modified from the method that was described by Mahanonda et al. (19). Briefly, the tissues were washed in RPMI 1640 medium and cut into small fragments (1–2 mm^3). The fragments were incubated in medium that contained 2 mg/ml collagenase type I (Life Technologies). After 90-min incubation (37˚C), residual tissue fragments were disaggregated by gentle flushing, until single-cell suspensions were obtained. These cells were filtered through filter (mesh size 70 μm).

Flow cytometric analysis of B cell subsets

To characterize the profiles of B cell subsets (naive B cells, memory B cells, ASCs) in isolated gingival cell suspension form, periodontal tissues and heparinized whole blood were stained with mouse anti-human CD19 (FITC), CD27 (PE), CD3 (PerCP), and CD38 (allophycocyanin) mAbs at 4˚C for 30 min, whereas whole blood was stained at room temperature for 30 min. Naive B cells, memory B cells, and ASCs were characterized as $CD19^{+}CD27^{-}CD38^{-}$, $CD19^{+}CD27^{+}CD38^{-}$, and $CD19^{+}CD27^{+}CD38^{+}$, respectively. The stained gingival cells were washed with stain buffer (BD Biosciences). The stained cells were treated with RBC lysing solution for 10 min at room temperature in the dark, washed, and then fixed with 1% paraformaldehyde. Analysis of flow cytometry samples was performed by four-color flow cytometry (FACSCalibur; BD Biosciences).

Differential expression between plasmablasts and plasma cells was used to investigate ASC subsets. Unlike plasmablasts and memory B cells, plasma cells express very low level of MHC class II (HLA-DR) (20–22) and also express CD138 (a well-known marker for plasma cell) (18, 23–25).

To identify surface IgG and IgA on memory B cells, gingival cell suspension form periodontal healthy tissues were stained with mouse anti-human IgG (PE) or IgA1/IgA2 (PerCP) mAbs. IgG memory B cells and IgA memory B cells were characterized as $CD19^{+}CD27^{+}CD38^{-}IgG^{+}$ and $CD19^{+}CD27^{+}CD38^{-}IgA1/IgA2^{+}$, respectively.

Immunohistochemistry

The excised periodontal tissues were immediately washed in normal saline solution. For paraffin-embedded sections, they were fixed in 10% buffered formalin for a maximum of 24 h and subsequently embedded in paraffin. Microtome serial 4-μm–thick sections were cut and mounted on glass slides. Sections were deparaffinized. To inhibit endogenous peroxidase, they were incubated with 0.3% hydrogen peroxide solution for 20 min, and heated in 1 mM EDTA (pH 8.0) at 95˚C for 20 min for Ag retrieval.

For identifying local plasma cells, single immunohistochemical staining was performed via polymer/HRP and diaminobenzidine $(DAB)^{+}$ chromagen system (DAKO EnVisionTM G/2 Doublestain System) on the sections. They were stained with primary mouse anti-human CD138 or isotype control. Counterstaining was done with hematoxylin. They were investigated under light microscope. To identify gingiva memory B cells, double immunohistochemical staining with mAbs to CD20 and CD27 was performed via polymer/HRP and DAB^{+} chromagen system on the sections. Expression of PNAd and ICAM-1 on gingival blood vessel endothelial cells, tissue staining with mAbs to PNAd, and ICAM-1 was performed via polymer/HRP and DAB^{+} chromagen system on the sections.

ELISPOT assay

To characterize Ag-specific Ab production, the frequency of Ag-specific ASCs from periodontal tissues was measured by ELISPOT assay. Briefly, Multiscreen 96-well filtration plates (Millipore) was coated with a variety of soluble bacterial Ags. These included key periodontal pathogens, as follows: 1) *P. gingivalis* ATCC33277; 2) *A. actinomycetemcomitans* Y4; 3) commensal plaque bacteria: *Streptococcus gordonii* DL-1 (from F. Yoshimura, Department of Microbiology, School of Dentistry, Aichi Gakuin University, Nagoya, Japan); and 4) self-tissue: type I collagen (Sigma-Aldrich) overnight at 4˚C in a humidified chamber. Keyhole limpet hemocyanin was used as a negative control.

The plate was washed twice with Dulbecco PBS (DPBS; Life Technologies) and blocked with DPBS containing 10% FBS for 1 h at 37˚C.

After being washed twice with DPBS, gingival mononuclear cells were added and incubated overnight at 37˚C. After that, the plate was washed three times with PBS and another three times with PBS with 0.05% Tween 20 (PBST). Goat anti-human IgG or IgA biotin conjugated (KPL) was added into the plate. After 2 h of incubation at 37˚C, the plate was washed four times with PBST, and streptavidin-alkaline phosphatase was added to the plate at a 1:1000 dilution and incubated for 1 h at 37˚C. Then the plate was washed three times with PBST and another three times with PBS; after that a substrate (5-bromo-4-chloro-3-indolyl phosphate/NBT; Sigma-Aldrich) was added to allow spots to develop for 5–15 min, and then spots were counted by using an ELISPOT reader (Cellular Technologies).

To investigate the ability to secrete IgG and IgA of the memory B cells, the gingival cell suspension from periodontal healthy tissue was activated with TLR7/8, R848 (Resiquimod; Innovagen), and human rIL-2 (R&D Systems) for 6 d in 37˚C, 5% CO_2. After 6 d of incubation, IgG- and IgA-secreting cells were detected by ELISPOT assay, as described above for plasma cells.

Real-time RT-PCR

To study the role of local tissue mediators in B cell response, total RNA from periodontal tissue samples was isolated by using an RNeasy mini kit from Qiagen. One microgram of DNase I–treated total RNA was reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR (Promega). The cDNA was then divided and used for PCR amplification of chemokines and survival factors for B cell responses. Real-time RT-PCR assays were performed on LightCycler System 480 (Roche Molecular Diagnostics) using SYBR Green PCR Master Mix (Roche Molecular Diagnostics). CXCL12, a proliferation-inducing ligand (APRIL), B cell–activating factor (BAFF), IL-10, IL-6, IL-21, and GAPDH were amplified using specific primers purchased from Operon. The PCR conditions have been described previously (26–29), and the primer sequences are shown as follows: CXCL12 (5′-ATGCCCATGCCGATTCTT-3′/5′-GCCGGGCTACAATCTGAAGG-3′), APRIL (5′-AGAGTCTCCCGGAGCAGAGTT-3′/5′-CTGGTTGCCACATCACCTCTGT-3′), BAFF (5′-TGAAACACCAACCTATACAAAAAG-3′/5′-TCAATTCATCCCCAAAGACAT-3′), IL-10 (5′-GGCGCTGTCATCGATTTCTT-3′/5′-TGGAGCTTATTAAAGGCATTCTTCA-3′), IL-6 (5′-CCTGAACCTTCCAAAGATGG-3′/5′-ACCAGGCAAGTCTCCTCATT-3′), IL-21 (5′-TGTGAATGACTTGGTCCCTGAA-3′/5′-ACCAGGAAAAAGCTGACCACTCA-3′), and GAPDH (5′-GAAGGCTGGGGCTCATTT-3′/5′-CAGGAGGCATTGCTGATGAT-3′). Amplification conditions, sequences, and concentrations of the primers were similar to those of RT-PCR. After 45 reaction cycles, the melting curve analysis was performed at 95˚C for 5 s, 65˚C for 1 min, and heating to 97˚C using a ramp rate of 0.11˚C/s with continuous monitoring of fluorescence. The melting peak generated represented the specific amplified product. All samples had only a single peak, indicating a pure product and no primer/dimer formation. Amplicons of a single band with the expected sizes were also confirmed in all reactions by agarose gel electrophoresis. The amplification efficiencies were high (close to 100%) when multiple standard curves were performed using serial mRNA dilutions.

For periodontal tissue specimens, the relative mRNA expression was normalized to corresponding GAPDH for each sample, using the formula = $2^{-\Delta CT}$, where $\Delta CT = C_{T-geneX} - C_{T-GAPDH}$. The relative quantification of mRNA expression in periodontitis tissues was presented as the mean fold increase ± SEM, using the mean value obtained from the healthy tissue as a reference (relative quantification = 1).

Intracellular staining analysis of plasma cell: RANKL and granzyme B

To investigate the expression of RANKL and granzyme B in plasma cells, gingival cells were first stained for surface-expressing molecules with anti-human CD19, CD27, and CD38 mAbs at 4˚C for 30 min. The stained gingival cells were washed with stain buffer and then fixed and permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences) on ice for 20 min and washed with BD Perm/Wash buffer (BD Biosciences). The cells were then suspended in BD Perm/Wash buffer, and anti-human RANKL or anti-human granzyme B or isotype control mAbs were added. After another 30 min of incubation on ice, cells were washed and then fixed with 1% paraformaldehyde. Analysis of flow cytometry samples was performed by four-color flow cytometry, FACSCalibur.

Statistical analysis

Results were expressed as mean ± SEM. Comparisons between multiple clinical groups were analyzed using the Kruskal–Wallis test by rank, and post hoc comparisons were made using the Mann–Whitney *U* test with Bonferroni's correction. Comparisons between multiple B cell subsets within each clinical group were analyzed using the Friedman test by rank, and post hoc comparisons were made using the Wilcoxon signed-rank test with Bonferroni's correction. Comparisons between two variables in one group were analyzed using Wilcoxon signed-rank test. In cases in which data were normalized to healthy control, a one-sample Wilcoxon signed-rank test was used with an expected value of 1. Comparisons of one variable between two groups were analyzed using the Mann–Whitney *U* test. Two-tailed p values <0.05 were considered statistically significant (SPSS version 22.0).

Results

Phenotypic characterization of B cell subsets in gingival tissues

A mixture of mAbs consisting of anti-CD19, anti-CD27, and anti-CD38 was employed to identify different subsets of B cells in gingival tissues from clinically healthy gingiva, gingivitis, and periodontitis. Naive B cells were characterized as $CD19^+CD27^-CD38^-$, memory B cells were characterized as $CD19^+CD27^+CD38^-$, and ASCs as $CD19^+CD27^+CD38^+$, respectively. Flow cytometry analysis of periodontal tissues showed very few naive B cells (<8%) in all stages of healthy or diseased tissues.

Memory B cells represented the majority in the $CD19^+$ B cell population (86.59% ± 1.29) in the clinically healthy gingiva and in gingivitis tissues (85.90% ± 2.67). In periodontitis tissues, however, the density of memory B cells was 37.67% ± 3.39. The difference in the density of memory B cells in healthy and gingivitis tissues compared with periodontitis tissue was statistically significant ($p < 0.01$, Kruskal–Wallis test by rank, and post hoc comparisons by Mann–Whitney *U* test with Bonferroni's correction).

In periodontitis tissue, ASCs were the major cell type in the $CD19^+$ B cell population (58.44% ± 3.79). The mean percentage of ASCs was significantly higher than that of memory B cells ($p < 0.01$, Friedman test by rank, and post hoc comparisons by Wilcoxon signed-rank test with Bonferroni's correction) (Fig. 1A). In contrast, in the healthy and the gingivitis tissues, the mean percentage of memory B cells was significantly higher than the mean percentage of ASCs (6.64% ± 1.19; 6.34% ± 1.98, respectively).

Unlike in gingival tissues, there were no differences in peripheral blood B cell subsets between the healthy or diseased stages. In contrast to the gingival tissues, peripheral blood-naive B cells represented a major population (>64 and 65% in healthy and periodontitis patients, respectively) in $CD19^+$ B cell population, followed by memory B cells (34 and 33% in healthy and periodontitis patients, respectively), and very small proportions of ASCs (2.1 and 2.0% in healthy and periodontitis patients, respectively) (Fig. 1B).

In addition, gingival tissues from periodontitis patients ($n = 6$) who received initial periodontal treatment of scaling and root planing (removal of biofilms) and who were scheduled for some tooth extractions were analyzed. As depicted in Fig. 1C, the majority of B cell subsets at treated sites represented high proportions of memory B cells similar to those of clinically healthy gingiva and gingivitis tissues.

Memory B cells in clinically healthy gingiva

Because high proportions of $CD19^+CD27^+CD38^-$ memory B cells among the $CD19^+$ B cell population were identified in clinically healthy gingiva (Fig. 1A), the anatomical compartmental location of this specific population in histological sections was investigated. Because CD19 staining in formaldehyde-fixed, paraffin-embedded tissues was not successful in our experiments, mAbs against CD20 and CD27 were used to identify memory B cells. A

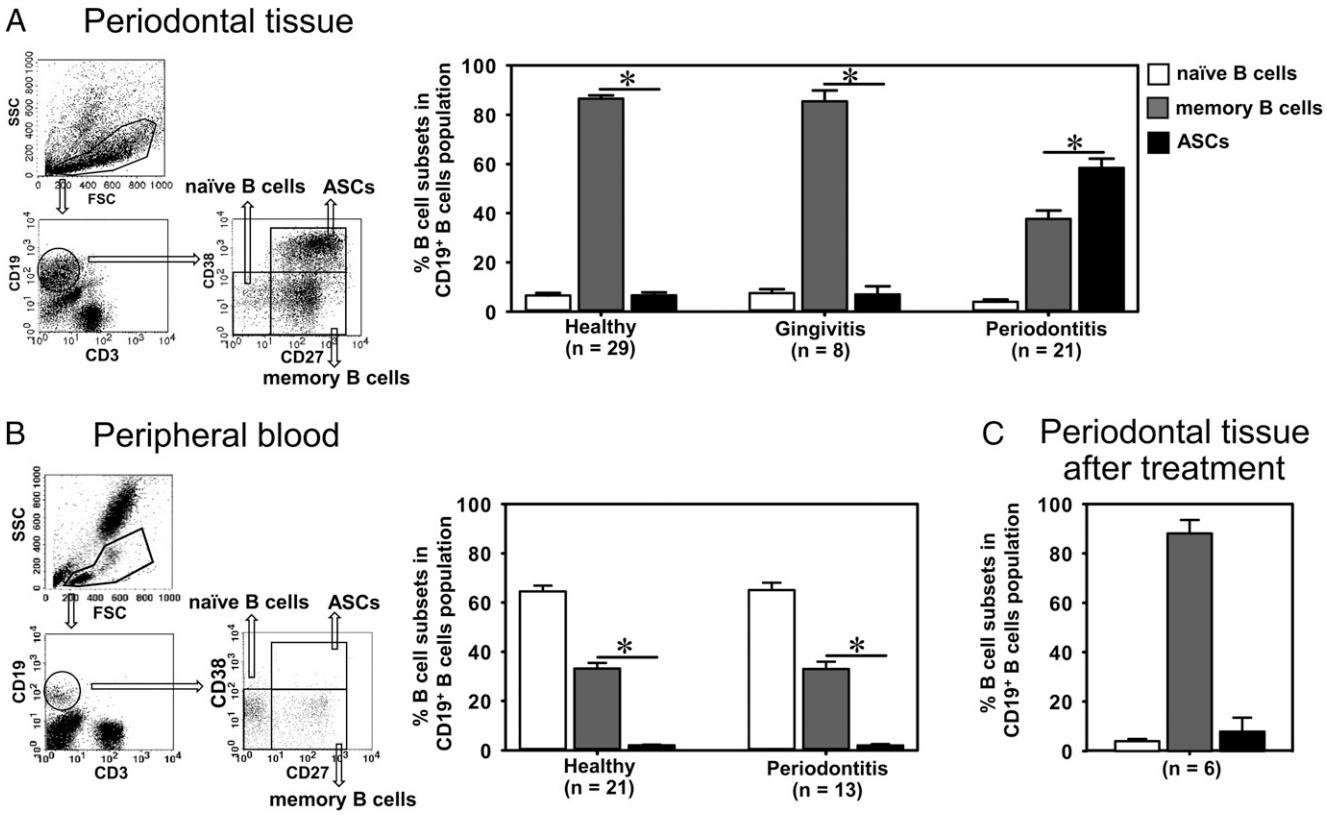


FIGURE 1. B cell subsets in gingival tissues and peripheral blood. Cells were extracted from (**A**) tissue specimens of clinically healthy gingiva ($n = 29$), gingivitis ($n = 8$), and periodontitis ($n = 21$), and (**B**) peripheral blood from subjects with clinically healthy gingiva ($n = 21$) and periodontitis ($n = 13$). (**C**) Treated periodontal tissues after scaling and root planing ($n = 6$). Naive B cells, memory B cells, and ASCs were classified as $CD19^+CD27^-CD38^-$, $CD19^+CD27^+CD38^-$, and $CD19^+CD27^+CD38^+$, respectively. Data shown are mean ± SEM. *$p < 0.01$ Wilcoxon signed-rank test with Bonferroni's correction.

cluster of $CD20^+CD27^+$ memory B cells in clinically healthy gingiva was detected in the connective tissue subjacent to the apical region of the junctional epithelium (Fig. 2A). A representative flow cytometry histogram in Fig. 2B demonstrates that gingival memory B cells from clinically healthy human gingiva also expressed surface IgG and IgA, thus confirming their characteristics of memory B cells. Moreover, it was explored whether these gingival tissue-memory B cells could be differentiated in vitro into ASCs. Upon polyclonal B cell activation with TLR7/8 (R848) and IL-2, these cells transformed into IgG and IgA ASCs, as assessed by ELISPOT assay (Fig. 2C). A significantly higher frequency of IgG spot-forming cells (SFCs) was observed when compared with IgA-SFCs ($n = 3$, $p < 0.05$, Wilcoxon signed-rank test). It should be pointed out that endothelial cells of the blood vessels in the connective tissue subjacent to the junctional epithelium of healthy gingiva expressed peripheral node addressin ($PNAd^+$) and $ICAM\text{-}1^+$ (Fig. 3). Inducible PNAd and ICAM-1 are known to bind with lymphocyte endothelial cell adhesion molecule-1 and LFA-1, respectively, on B cells (30, 31).

Phenotypic characterization of ASC subsets in periodontal tissues

$CD19^+CD27^+CD38^+$ ASCs consist of two subsets: plasmablasts and plasma cells (20, 32). Unlike plasmablasts, plasma cells express very low level of HLA-DR (20, 33, 34) as compared with memory B cells. The levels of HLA-DR expression were then used to differentiate the two subpopulations of ASCs in periodontal tissues. It was found that ASCs in periodontitis tissues expressed ~7-fold lower levels of HLA-DR (mean fluorescence intensity [MFI] 37.62 ± 7.54, $n = 10$) than memory B cells (mean MFI 271.19 ± 33.06, $n = 10$, $p < 0.05$, Wilcoxon signed-rank test) (Fig. 4A). These $CD19^+CD27^+CD38^+HLA\text{-}DR^{low}$ cells were also positive for CD138 (syndecan-1), a well-known plasma cell marker (35) (Fig. 4A). Memory B cells were in very low levels for the CD138 expression. In contrast, circulating ASCs in peripheral blood showed a high expression level of HLA-DR (mean MFI was 229.47 ± 31.46, $n = 5$), a phenotypic marker of plasmablasts (data not shown).

Subsequently, the anatomical compartmental location of plasma cells in periodontal tissues was examined by immunostaining using anti-CD138 Ab. Paraffin-embedded sections prepared from gingival tissue specimens were obtained from clinically healthy gingiva, gingivitis, and periodontitis (Fig. 4B–D). Anti-CD138 Ab is known to react not only with plasma cells, but also with gingival epithelium (36). An abundance of $CD138^+$ plasma cells was observed in periodontitis tissues as compared with gingivitis and clinically healthy gingiva. Plasma cells were arranged in clusters that were detected at the base of the periodontal pocket area and scattered throughout the gingival connective tissue, especially apically toward the advancing front of the lesion (Fig. 4D). Small numbers of plasma cells were identified in gingivitis and, to a much lesser extent, in clinically healthy gingiva (Fig. 4B, 4C). A few smaller clusters of plasma cells were detected in the connective tissue subjacent to the junctional epithelium of gingivitis tissue, whereas they were loosely scattered through the gingival connective tissue in healthy tissues (Fig. 4B).

Molecules involved in B cell responses in periodontal tissues

The expressions of CXCL12, BAFF, APRIL, IL-10, IL-6, and IL-21 molecules that are involved in B cell recruitment, survival, and differentiation (37) were assessed in periodontal tissues. The

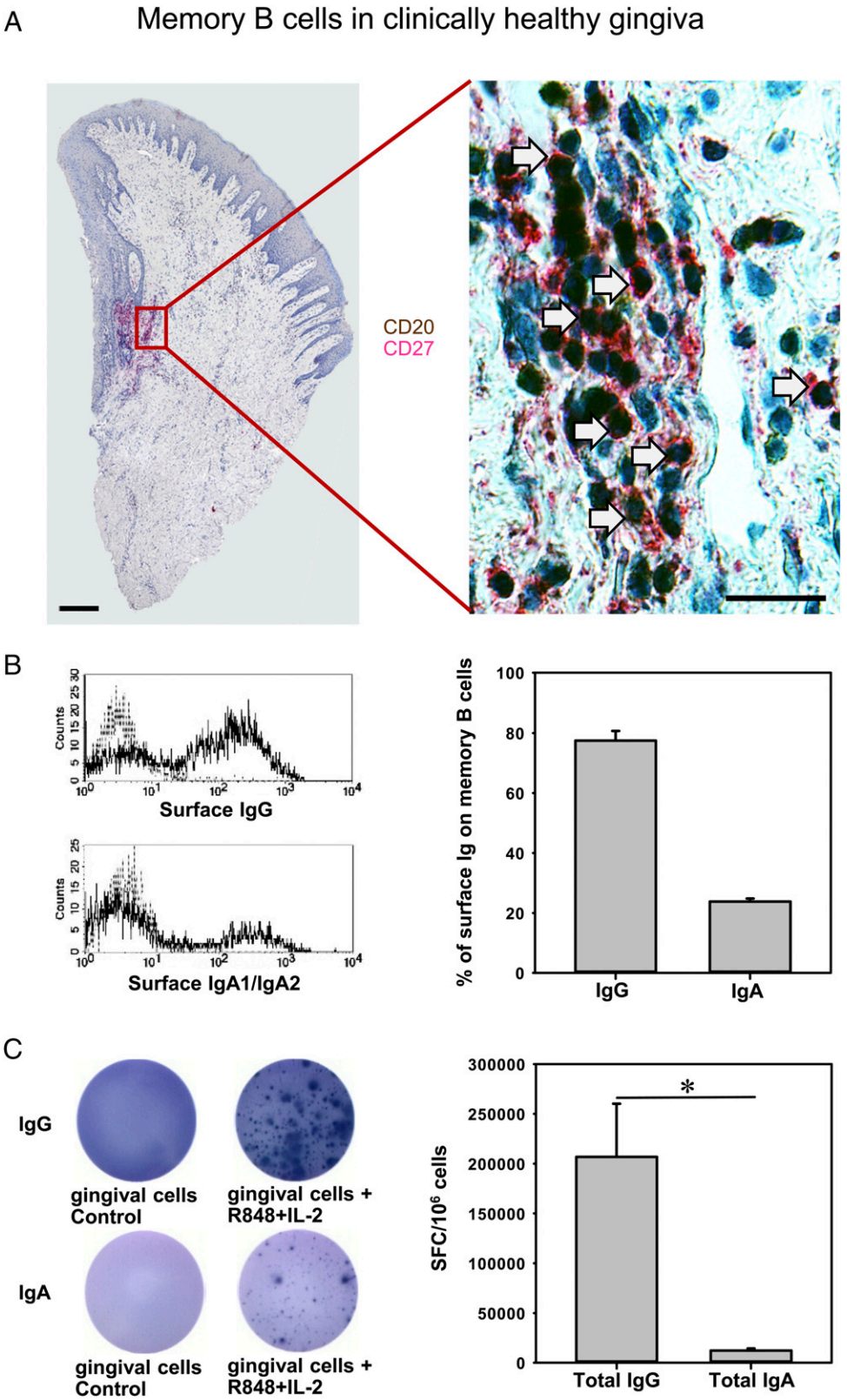


FIGURE 2. Anatomical compartmental location and characterization of memory B cell in clinically healthy gingival tissue. (**A**) Representative immunostaining of memory B cells from three different healthy individuals. Memory B cells were identified as $CD20^+CD27^+$ B cells. (**B**) Representative of surface expression of IgG and IgA on $CD19^+CD27^+$ memory B cells by flow cytometry from three different healthy individuals. Data shown are mean ± SEM. (**C**) Representative of IgG and IgA ELISPOT assay and the frequency of SFC after stimulation with R848 and IL-2 from three different healthy individuals. Data shown are mean ± SEM ($*p < 0.05$, Wilcoxon signed-rank test). Scale bars, 200 μm.

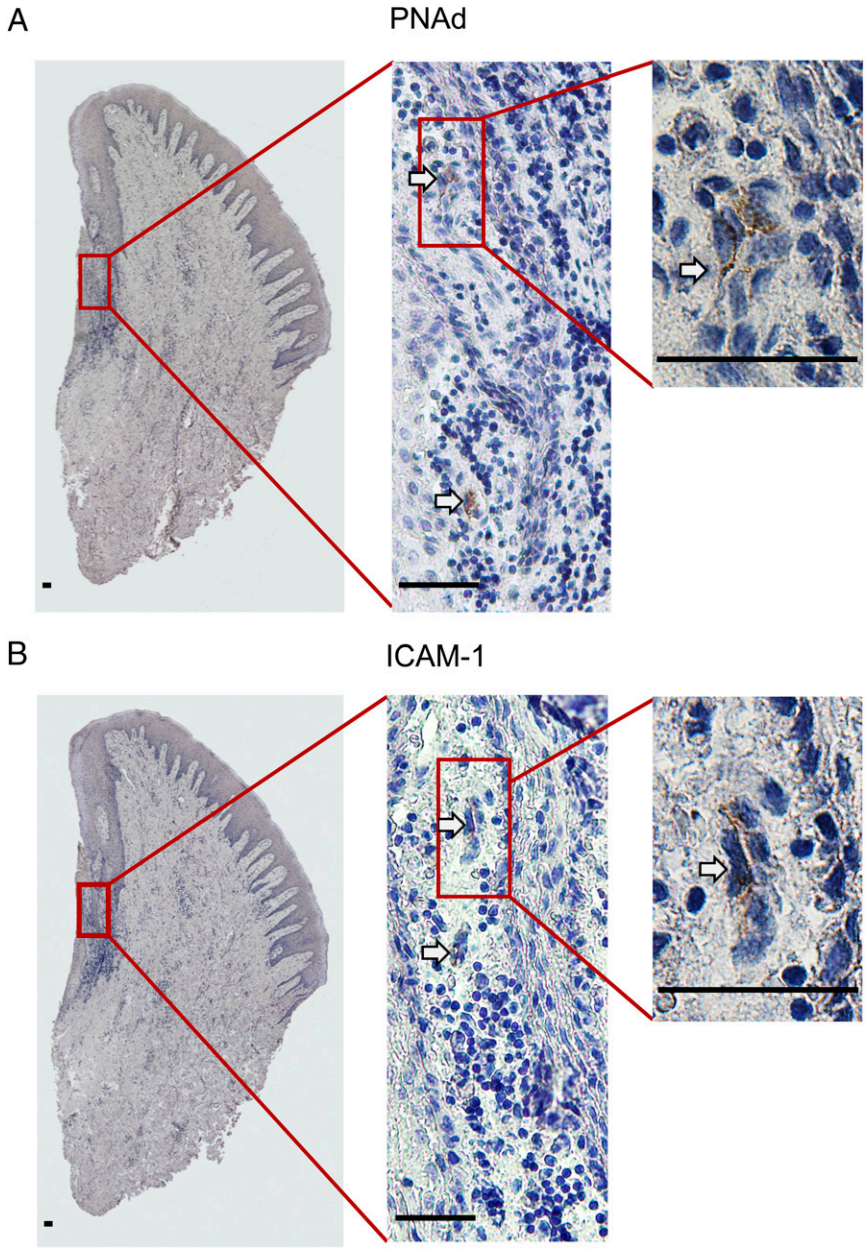


FIGURE 3. Expression of PNAd and ICAM-1 in clinically healthy gingiva. Representative immunostaining of endothelial PNAd (**A**) and ICAM-1 (**B**) in the connective tissue subjacent to the junctional epithelium from three different healthy subjects. Scale bars, 40 μm.

relative quantification expressed as fold change for each studied molecule is shown in Fig. 5. Although mRNA expression for CXCL12, APRIL, BAFF, IL-10, IL-6, and IL-21 was detected in all tissue specimens of clinically healthy gingiva, gingivitis, and periodontitis, a significant overexpression of IL-21 was detected in periodontitis tissues compared with clinically healthy gingiva (34-fold, $p < 0.01$, one-sample Wilcoxon signed-rank test). Similarly, CXCL12 (4-fold), APRIL (3-fold), BAFF (4-fold), and IL-10 (3.8-fold) were significantly overexpressed in periodontitis tissues when compared with healthy gingiva ($p < 0.01$, one-sample Wilcoxon signed-rank test). In addition, a significant overexpression of CXCL12 (9-fold) and IL-6 (21-fold) was detected in gingivitis tissues compared with healthy gingiva ($p < 0.01$, one-sample Wilcoxon signed-rank test). Even though gingivitis tissues highly expressed IL-21 expression (23-fold), no significant difference was found, and this may be due to a large variation in each sample. Moreover, there were no significant differences between periodontitis and gingivitis in all expressions ($p > 0.05$, Mann–Whitney U test).

Ag specificity and expression of RANKL and granzyme B of plasma cells in periodontitis tissues

Ag specificity of plasma cells in periodontitis tissues was evaluated using ELISPOT assays. Fig. 6A shows SFCs specific to *P. gingivalis*, *A. actinomycetemcomitans*, *S. gordonii*, and collagen type 1 in representative ELISPOT analyses. Plasma cells producing IgG and IgA to *P. gingivalis* soluble Ags in all five tissue specimens were consistently detected (Fig. 6B). The frequency of *P. gingivalis*-specific plasma cells producing IgG isotype (mean SFCs = 4532 ± 1908/10^6 input mononuclear immune cells; $p < 0.05$, Wilcoxon signed-rank test) was significantly higher compared with IgA isotype (mean SFCs = 842 ± 753/10^6 input mononuclear immune cells). Plasma cells producing IgG isotype specific *to A. actinomycetemcomitans* soluble Ags were detected in three of five specimens with mean frequencies of 78 ± 63/10^6 input mononuclear immune cells. *A. actinomycetemcomitans*-specific plasma cells producing IgA isotype were not detected (under assay sensitivity). The presence of plasma cells specific to commensal bacteria, *S. gordonii*,

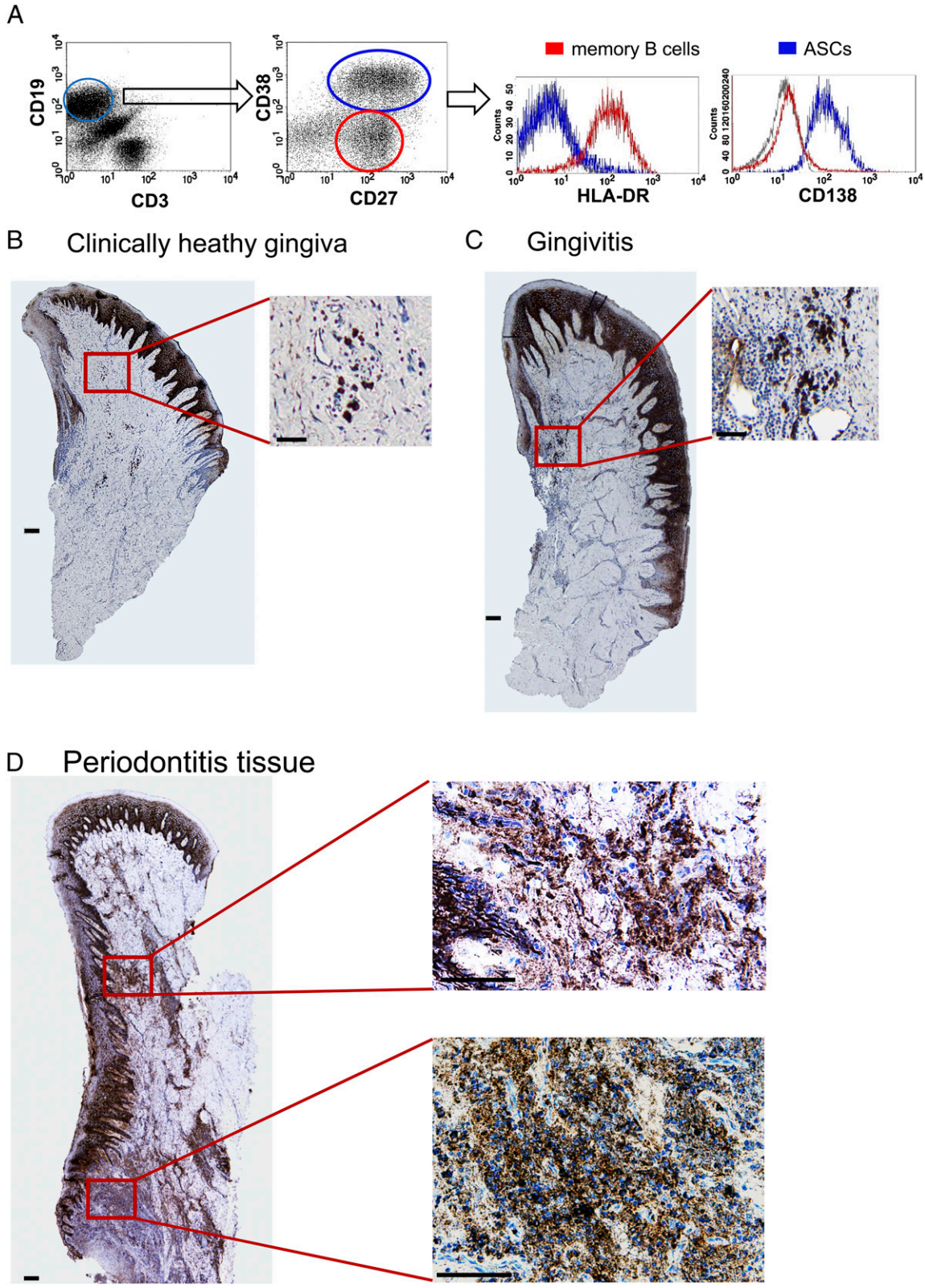


FIGURE 4. Phenotypic and anatomical location of ASCs in gingival tissues. (**A**) Flow cytometric analysis of HLA-DR and CD138 expression on ASCs isolated from periodontitis tissues ($n = 10$). Black line in histograms represents isotype control, and red and blue lines represent memory B cells and ASCs, respectively. Data are representative of 10 different individuals in each clinical group. Immunostaining of $CD138^+$ plasma cells in tissue specimens from (**B**) clinically healthy gingiva ($n = 7$) (**C**), gingivitis ($n = 3$), and (**D**) periodontitis ($n = 11$). Data are representative of different individuals in each clinical group. Scale bars, 100 μm.

and collagen type 1 was immeasurable. Keyhole limpet hemocyanin protein-coated well was used as a negative control. Negligible numbers of SFCs in the keyhole limpet hemocyanin controls were consistently detected, suggesting a high specificity of the assay system.

To further extend the analysis of the possible role of periodontitis plasma cells in the pathogenesis process, the expression of RANKL, a key molecule in bone-resorbing activity, was assessed (38). RANKL could be expressed as a preform intracellular and

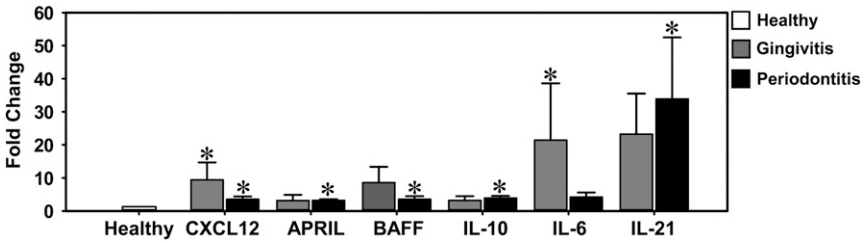


FIGURE 5. mRNA expression of molecules involved in B cell responses in gingival tissues. Tissue specimens from clinically healthy gingiva (n = 10), gingivitis (n = 7), and periodontitis (n = 10) were used for real-time RT-PCR for CXCL12, BAFF, APRIL, IL-10, IL-6, and IL-21 expression. The relative quantification of mRNA for each molecule in periodontitis tissues and gingivitis was obtained considering the normalized expression in clinically healthy gingiva as reference (relative quantification = 1). One tissue specimen per experiment and each specimen derived from different individuals. Data shown are mean ± SEM. *$p < 0.01$, compared with healthy group, one-sample Wilcoxon signed-rank test.

cell surface protein (39). As shown in Fig. 6C, both cell surface and intracellular RANKL were detected on periodontitis plasma cells. The cell surface expression of RANKL was modest and consistently lower than that of the intracellular RANKL (n = 3). Moreover, the intracellular expression of granzyme B, a molecule that can degrade extracellular matrix (40), was measured with no detectable expression.

Discussion

The present study has identified various subsets of B cells in human periodontal tissues obtained from clinically healthy, gingivitis, and periodontitis sites. Moreover, gingival biopsies were analyzed from patients that had suffered from periodontitis and were subsequently treated for the disease. This resulted in a resolution of the disease characteristics. Hence, the presence of various subsets of B cells could be clearly studied and their localization within the marginal gingival tissues enumerated in various conditions ranging from clinical health to periodontitis and reversed to treated periodontitis.

The most intriguing findings in the current study were the identification of $CD19^+CD27^+$ memory B cells in human clinically healthy gingiva. Unlike tissue-resident memory T cells, very little is known about memory B cells residing in human nonlymphoid tissues. The evidence is limited to a few reports of tissue memory B cells in healthy nonlymphoid tissues in animals, such as in the lung of mice after influenza infection (41). Other studies described the presence of B cells within adventitia of normal aortas in mice (42). Up to date, only one report dealt with the presence of memory B cells in normal human skin (43).

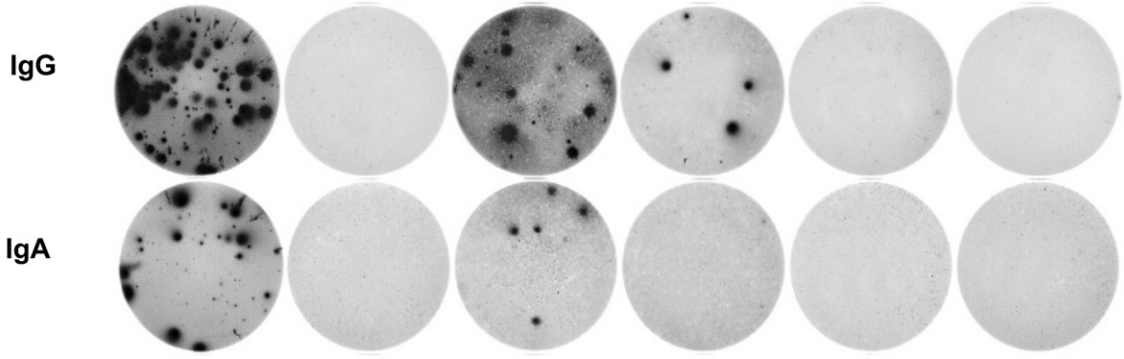
The observed memory B cells in human clinically healthy gingiva preferentially resided in the connective tissue subjacent to the junctional epithelium. In this vicinity, $PNAd^+$ and $ICAM\text{-}1^+$ were detected on blood vessels, suggesting a local low-grade inflammatory response due to constant challenge of the bacterial biofilm in the sulcular area. One could speculate that endothelial PNAd and ICAM-1 may be responsible for a sustained recruitment of memory B cells in clinically healthy gingiva. This memory B cell trafficking may take place along with the well-documented polymorphonuclear cell emigration, a well-recognized phenomenon in a clinically healthy sulcus (44, 45). Such gingival memory B cells were obviously functional, as they were able to secrete IgG and IgA after in vitro polyclonal stimulation in the current study. However, questions remain still unanswered about their role in the homeostasis of clinically healthy gingiva. For instance: 1) how did these memory B cells emigrate from the blood circulation and reside subjacent to the junctional epithelium; 2) what was the Ag specificity of these memory B cells; 3) did they, at a later stage, differentiate into Ab-secreting plasma cells that were found in large numbers in periodontitis; and 4) is there a role of these memory B cells in first line of defense to maintain gingival homeostasis?

Massive infiltrated B cells and plasma cells have been recognized in inflamed tissues of advanced periodontitis (46–48). Although such features have been deep rooted in the vision of periodontitis pathogenesis, current knowledge of B cell responses in the diseased tissues has been limited. In the current study, previous reports of the dominance of B cells in periodontitis lesions (49, 50) have been confirmed. Owing to the fact that ASCs in periodontitis tissues expressed ~7-fold lower levels of HLA-DR than memory B cells, it must be assumed that the observed majority of the ASCs in diseased tissues were actually plasma cells ($CD19^+CD27^+CD38^+CD138^+HLA\text{-}DR^{low}$), rather than plasmablasts ($CD19^+CD27^+CD38^+CD138^-HLA\text{-}DR^{high}$). Aggregates of plasma cells were found at the base of the periodontal pockets and scattered in periodontal connective tissue of marginal gingiva, especially apically toward the advancing front of the lesion. Unlike in periodontitis lesions, significant proportions of memory B cells ($CD19^+CD27^+CD38^-$) were detected in both clinically healthy gingiva and gingivitis tissues. Yet, very low numbers of naive B cells ($CD19^+CD27^-CD38^-$) were enumerated in all clinical tissue specimens without any statistically significant differences. It remains unclear whether the observed naive B cells come from blood contamination or represent cells that truly reside in the tissue. The B cell subsets in gingival tissues obviously differ from those in peripheral blood of corresponding subjects. In the peripheral blood, two thirds of the B cells belonged to the subset of naive B cells. It should be pointed out that the number of follicular Th cells ($CD4^+PD\text{-}1^+CXCR3^+CXCR5^+$), which are critical for germinal center formation, was negligible in periodontitis tissue (data not shown). The data are in line with previous study, which argues against the formation of germinal center in periodontitis tissue by showing the lack of reticular organization by follicular dendritic cells (50).

Periodontal therapy for patients with periodontitis involving scaling and root planing (removal of the plaque biofilm) resulted in a reversal shift of the tissue B cell profile resembling closely that of the B cell response in clinically healthy and gingivitis tissues. Again, the majority of the B cells were memory B cells, and not plasma cells. These findings documented that the bacterial plaque biofilm was responsible for the induction of local B cell response in periodontitis tissues.

High expression of known molecules involved in B cell response was detected in diseased tissues. Among all expressions, IL-21 was the highest in gingivitis and periodontitis lesions. IL-21 has a variety of actions that influence on B cell response and its destiny (51). The major source of IL-21 is follicular Th cells (52) and Th17 cells (53). Very low numbers of $CD4^+PD1^+CXCR5^+$ follicular Th cells were identified in the periodontitis tissues in the

A



B

	P. gingivalis		A. a		S. gordonii		Collagen I	
	n	SFCs/10^6 cells	n	SFCs/10^6 cells	n	FCs/10^6 cells	n	SFCs/10^6 cells
IgG	5/5	4,532±1,908	3/5	78±63	0/5	-	0/5	-
IgA	5/5	842±753	0/5	-	0/5	-	0/5	-

*

C

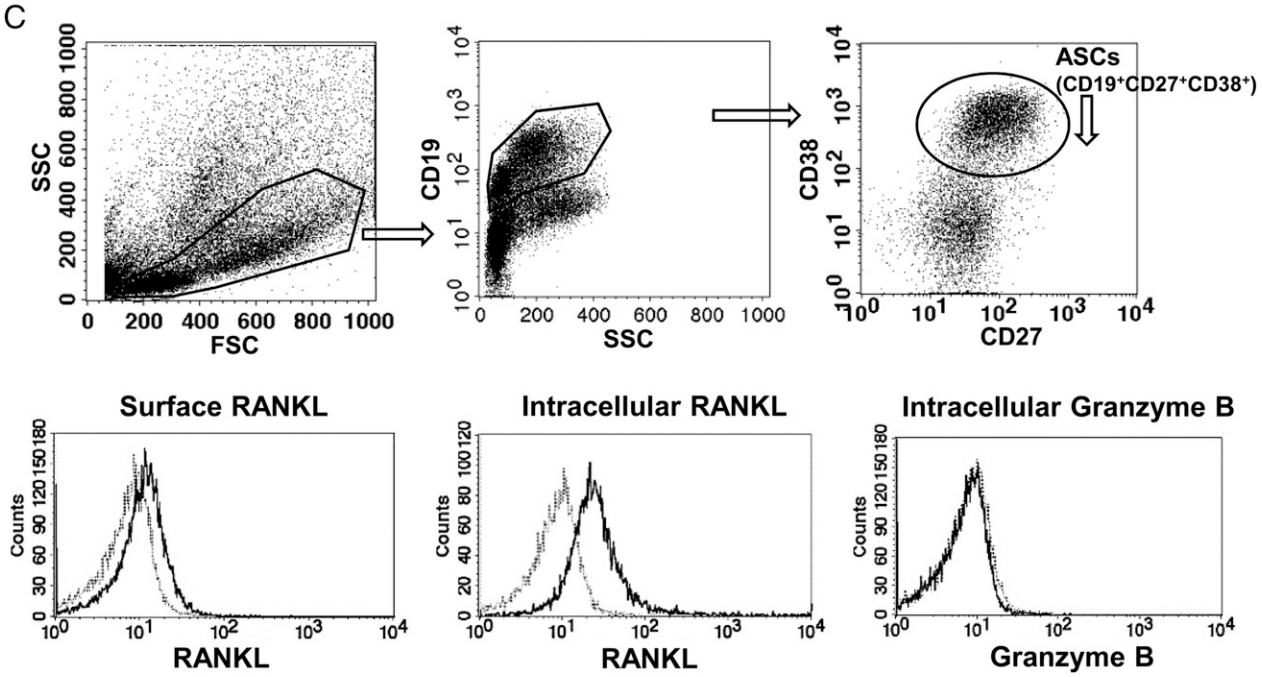


FIGURE 6. Ag specificity and expression of RANKL and granzyme B of plasma cells. The numbers of SFCs specific to *P. gingivalis*, *A. actinomycetemcomitans* (*A.a*), *S. gordonii*, or collagen type 1 were assessed on cells extracted from periodontitis tissues. (**A**) Representative ELISPOT analyses of SFCs specific to *P. gingivalis*, *A. actinomycetemcomitans*, *S. gordonii*, or collagen type 1 in periodontitis tissue specimens from five different individuals. Keyhole limpet hemocyanin was used as a negative control. (**B**) Numbers of SFC with different Ag specificity. Data shown are mean ± SEM. *$p < 0.05$, Wilcoxon signed-rank test. (**C**) Expression of RANKL (surface and intracellular staining) and granzyme B (intracellular staining) on plasma cells. Dotted lines represent isotype control, and solid lines represent RANKL or granzyme B. Data are representative of periodontitis tissue specimens from three different individuals.

current study (<1%, $n = 4$, data not shown). This suggests that IL-21 may be derived from other cell types such as Th17 cells. Recent studies demonstrated the presence of Th17 cytokine in periodontitis tissues (9, 54). However, the ability of this T cell subset in producing IL-21 in periodontitis requires further investigation. Despite the high expression of IL-21 in gingivitis, plasma cell numbers were low compared with the periodontitis lesion. It is well established that IL-21 induces greater plasma cell differentiation in the presence of CD40 signaling (55). It may be speculated that large infiltration of activated T cells expressing CD40L in periodontitis, but not in gingivitis, may increase CD40 signaling on B cells, leading to enhancement of plasma cell differentiation.

Periodontitis tissue plasma cells in all specimens secreted Abs specific to *P. gingivalis* and, to a lesser extent, to *A. actinomycetemcomitans*. No commensal *S. gordonii*-specific Ab or tissue collagen type 1–specific Ab was detected. The data of the current study confirm those of earlier studies for the presence of different Ig isotypes specific for key pathogens in periodontitis tissue (56, 57). The presence of a large infiltrate of plasma cells and with constitutive high levels of Abs specific to pathogens does not seem to eliminate pathogens and hence stop the progression of disease. One explanation may be the nature of the plaque biofilm that made the biofilm more resistant to Ab-mediated protection. *Staphylococcus epidermidis* within the biofilm was found to be more resistant to opsonic killing mediated by Ab compared with a planktonic form of *S. epidermidis* (58).

It should be pointed out that the possible deposition of large Ag–Ab complexes may activate the complement classical pathway and neutrophils, resulting in periodontal tissue destruction (59). Deposits of Igs, complements, and the formation of immune complexes in inflamed human gingiva are involved in the acute phase of periodontal destruction in rats (60).

Recently, it has been shown that most $CD20^+$ B cells in periodontitis lesions express RANKL (61). However, it is unclear whether those cells are plasma cells or memory B cells. In the current study, it has been shown that $CD19^+CD27^+CD38^+$ plasma cells, the major B cell component in periodontitis tissues, expressed RANKL. Moreover, gut plasma cells in patients with inflammatory bowel disease were demonstrated to be positive for granzyme B (62). However, no granzyme B–positive plasma cells were detected in periodontitis tissues.

In conclusion, the current study detected large proportions of memory B cells residing subjacent to the junctional epithelium in clinically healthy gingiva and gingivitis. Unlike tissue-resident memory T cells, the understanding of human B cell immunology in tissues has been very limited. Moreover, the presence of numerous plasma cells in periodontitis tissue and, to a much lesser extent, in gingivitis and clinically healthy gingiva was confirmed in the current study. These periodontitis plasma cells secreted IgG and IgA to *P. gingivalis* and *A. actinomycetemcomitans* and also expressed bone-resorbing cytokine, RANKL. Gingival tissues from both healthy and diseased sites obtained during dental procedures represent invaluable samples to study local human B cell immunology. Furthermore, the role of these cells in homeostasis and disease development of periodontal tissue may serve as a model for studying other chronic inflammatory diseases.

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Disclosures

The authors have no financial conflicts of interest.

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Tissue Distribution of Memory T and B Cells in Rhesus Monkeys following Influenza A Infection

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Studies of influenza-specific immune responses in humans have largely assessed systemic responses involving serum Ab and peripheral blood T cell responses. However, recent evidence indicates that tissue-resident memory T (T_{RM}) cells play an important role in local murine intrapulmonary immunity. Rhesus monkeys were pulmonary exposed to 2009 pandemic H1N1 virus at days 0 and 28 and immune responses in different tissue compartments were measured. All animals were asymptomatic postinfection. Although only minimal memory immune responses were detected in peripheral blood, a high frequency of influenza nucleoprotein–specific memory T cells was detected in the lung at the "contraction phase," 49–58 d after second virus inoculation. A substantial proportion of lung nucleoprotein-specific memory $CD8^+$ T cells expressed CD103 and CD69, phenotypic markers of T_{RM} cells. Lung $CD103^+$ and $CD103^-$ memory $CD8^+$ T cells expressed similar levels of IFN-γ and IL-2. Unlike memory T cells, spontaneous Ab secreting cells and memory B cells specific to influenza hemagglutinin were primarily observed in the mediastinal lymph nodes. Little difference in systemic and local immune responses against influenza was observed between young adult (6–8 y) and old animals (18–28 y). Using a nonhuman primate model, we revealed substantial induction of local T and B cell responses following 2009 pandemic H1N1 infection. Our study identified a subset of influenza-specific lung memory T cells characterized as T_{RM} cells in rhesus monkeys. The rhesus monkey model may be useful to explore the role of T_{RM} cells in local tissue protective immunity after rechallenge and vaccination. ***The Journal of Immunology*****, 2015, 195: 4378–4386.**

Influenza remains a global health problem with high degree of morbidity and mortality in young children and the elderly. Seasonal influenza vaccines, either trivalent inactivated or live attenuated influenza vaccines provide only moderate protection in adults and children with efficacy ranging from 59 to 83% (1). New improved influenza vaccines are needed to further reduce influenza-related morbidity and mortality. Serum hemagglutination-inhibition (HAI) titers against influenza viruses have been commonly used as correlates for protection (2) and serve as markers for designing influenza vaccines to induce strain-matched HAI Ab responses. These Abs are specific to the immunodominant globular domain of hemagglutinin (HA), thereby inhibiting binding of the virus to receptor on host target cells. It is well recognized that seasonal influenza vaccines do not confer protection on all vaccinated individuals. Some individuals with high HAI titers can be infected with influenza virus, whereas in others, clinical protection can be detected in the absence of HAI titers (3, 4) therefore suggest a role of cell-mediated immunity in protection.

Both natural infection and immunization with influenza A vaccines provide complete protection against reinfection with homologous virus. This is termed homotypic immunity. In contrast, heterosubtypic immunity is defined as immunity to an influenza subtype (i.e., heterologous influenza A virus that has a major change in the surface proteins [antigenic shift]). There is strong evidence in animal models that influenza-specific cross-reactive memory T cells are responsible for inducing heterosubtypic immunity (5–7). However, in humans, the role of cross-reactive memory T cells in protecting against influenza is not well elucidated. A recent human influenza challenge study demonstrated that the preexisting $CD4^+$ T cell responses to conserved nucleoprotein (NP) and matrix protein could reduce severe illness in the absence of specific Abs (8). In another study, a higher frequency of preexisting $CD8^+IFN\text{-}\gamma^+IL\text{-}2^-$ cross-reactive memory T cells against conserved core proteins (NP, M1, and PB1) in peripheral blood was associated with reduced severity of disease in humans infected with 2009 pH1N1 influenza (9), although this could reflect spillover of the responses initially generated in respiratory tract-draining lymph nodes.

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Abbreviations used in this article: ASC, Ab-secreting cell; CI, confidence interval; DC, dendritic cell; GMT, geometric mean titer; HA, hemagglutinin; HAI, hemagglutination-inhibition; iBALT, induced BALT; KLH, keyhole limpet hemocyanin; MDCK, Madin–Darby canine kidney; NP, nucleoprotein; PD-1, programmed death-1; SFC, spot-forming cell; $TCID_{50}$, 50% tissue culture infective dose; T_{RM}, tissue-resident memory.

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Viral infection and replication take place in the respiratory epithelial cells, yet most studies on influenza-specific memory T and B cells in humans have been conducted on immune cells isolated from peripheral blood, which may not reflect local lung immune responses. The role of local immunity has received more attention lately primarily because of the discovery of a new subset of memory T cells termed T_{RM} cells. These long-lived nonrecirculating T_{RM} cells permanently reside in nonlymphoid tissues including skin, brain, vagina, and lung and provide rapid, effective local protection against reinfection relative to circulating counterpart memory T cells (10, 11). Local immune protection by T_{RM} cells has been consistently documented in murine models of virus and bacterial infections including vaccinia virus, lymphocytic choriomeningitis virus, HSV, influenza, and tuberculosis (12–16). Recent studies have revealed delivery of vaccinia virus in mice via skin scarification generates long-lived skin T_{RM} $CD8^+$ T cells associated with improved long-term immunity (12, 17). In humans, protection against smallpox was first shown in 1796 by Edward Jenner using skin scarification with cowpox, ultimately leading to the eradication of the disease. Intravaginal heterologous prime/boost vaccination with human papillomavirus vectors (human papillomavirus pseudoviruses) expressing HSV Ags in mice preferentially induces cervicovaginal T_{RM} $CD8^+$ T cells and controls genital disease and virus shedding, whereas i.m. injection preferentially induces serum Ab response but fails to control either one (18). Targeting lung dendritic cells (DCs) via intranasal vaccination with Ag coupled to mAbs against endocytic receptors on DCs induces lung T_{RM} $CD8^+$ T cells that protect against lethal influenza challenge (19). Collectively, these data suggest that site of vaccine Ag priming could influence the localization of T_{RM} cells which are crucial for local host defense.

Most information about tissue distribution of memory T and B cells has been generated from a mouse model of microbial infection or vaccination (20–23). Rhesus monkeys are phylogentically close to humans and similarities of T and B cell immune responses in both species have been reported (24–27). Furthermore, TLR expression on Ag presenting DCs is similar in primates and humans (28). Rhesus monkeys have helped predict subsequent human immunogenicity of various formulations of malaria vaccines and such preclinical studies have contributed to development of the soon-to-be licensed RTS,S malaria vaccine (29, 30).The confirmation of comparable distribution pattern of memory T and B cells, resident memory cells in particular, subsets in rhesus monkeys and humans will greatly aid in designing vaccination strategies capable of inducing tissue-specific immunity. Rhesus monkeys have been previously employed to study immune response and immunopathology to 2009 pH1N1 (31-33). In this paper, we report the tissue distribution of memory T, B, and spontaneous Ab-secreting cells (ASCs) following infection with 2009 pH1N1influenza virus in rhesus monkeys as well as correlations between age and immune responses.

Materials and Methods

Animals and virus inoculations

All animal procedures were approved by the institution animal care and use committee in a facility accredited by the American Association of Laboratory Animal Care, incompliance with the Animal Welfare Act and Guide for the Care and Use of Laboratory Animals and in accordance with all applicable U.S. Department of Agriculture, Office of Laboratory Animal Welfare and Department of Defense guidelines. A stock of A/California/04/2009 (H1N1) virus was produced on Madin–Darby canine kidney (MDCK) cells and titrated using 50% tissue culture infective dose ($TCID_{50}$) assay. A total of 20 healthy Indian rhesus monkeys (*Macaca mulatta*) with no preexisting immunity to 2009 pH1N1 were selected and divided into two groups: control ($n = 4$) and infected group ($n = 16$). In the infected group, animals were subdivided into young adult (6–8 y of age; $n = 8$) and old monkeys (18–28 y of age; $n = 8$). Animals were inoculated twice with A/California/04/2009 (H1N1) virus at days 0 and 28, each inoculation consisted of 2×10^6 $TCID_{50}$ in 2 ml Leibovitz L-15 medium (L-15) with 1 ml administered intranasally (0.5 ml/nostril) and 1 ml intratracheal (Fig. 1). Nasal swabs were collected on days 2, 4, and 7 after each virus inoculation using Copan Flocked swabs and Copan universal transport medium (Copan Diagnostic, Brescia, Italy). Animals in control group were inoculated with L-15 medium. Virus replication in nasal secretions was assessed using a standard plaque assay. Briefly, confluent monolayers of MDCK were inoculated with 10-fold dilutions of samples at 37˚C for 1 h. The inoculums were removed, and cells were washed and overlaid with MEM containing 0.6% agarose and 0.2% serum albumin. After 2 d at 37˚C, cells were fixed in 37% formaldehyde solution for 1 h before stained with 1.25% crystal violet and plaque numbers (PFU/ml) were evaluated.

Tissue collection and cell separation

To evaluate memory T and B cell response, half of animals in each group were euthanized between days 14 and 23 and the other half between days 49 and 58 after second virus inoculation. Blood was collected before euthanasia and processed for PBMC by centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). The lung, mediastinal lymph nodes, spleen, and bone marrow were also harvested immediately after euthanasia. Mediastinal lymph nodes, spleen, and bone marrow were homogenized between the frosted ends of two slides, whereas lung tissues were cut into small pieces and homogenized in the presence of 1 μg/ml collagenase type 1 (Life Technologies, Grand Island, NY) using automated tissue dissociation (Miltenyi Biotec, Auburn, CA) and further incubated at 37˚C for another 1 h. Digested lung cells and cells derived from mediastinal lymph nodes, spleen, and bone marrow were then passed through 70-μm cell strainer (BD Falcon, Durham, NC). Then, single-cell suspensions were centrifuged using Histopaque-1077 to obtain mononuclear immune cells. They were treated with ammonium chloride–based lysing solution (BD Biosciences, San Jose, CA) if a large amount of RBCs were found to be contaminated in cell preparations. PBMC and mononuclear immune cells derived from different tissues were frozen in liquid nitrogen until use.

ELISA

IgG and IgA specific to influenza HA in nasal secretions were measured by ELISA. Briefly, 96-well ELISA plates (Dynex, Chantilly, VA) were coated with recombinant HA derived from A/California/04/2009 (H1N1) (0.5 μg/well) at 4˚C overnight and then washed with PBS containing 0.1% Tween 20 (Sigma-Aldrich). After blocking with 1% BSA in PBS with 0.1% Tween 20, nasal swab fluids (1:4 dilution) were added into coated, blocked plates and incubated for 2 h. Abs specific to HA were detected by anti-monkey IgG (Sigma-Aldrich) or IgA (KPL, Gaithersburg, MD) conjugated with peroxidase and developed with substrate using equal parts of solution A (2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate)) and B (H_2O_2) (KPL). The reaction was stopped with 5% $NaDodSO_4$ stop solution, and the plates were read at 405 nm on a SPECTRAmax plate reader (Molecular Devices).

HAI assay

Monkey serum samples were treated with receptor-destroying enzyme and subsequently heat-inactivated (30 min at 56˚C). The HAI assay was performed by World Health Organization standard methods using 8 HA units of influenza virus. Samples were tested in serial 2-fold dilutions by starting at 1:10 dilution. They were mixed with virus and incubated for 15 min at room temperature and then 50 μl of a 0.5% suspension of goose RBCs was added. The Ab titers were defined as the reciprocal of the highest dilution of sera samples that completely inhibited hemagglutination. Titers that were lower than the detection limit were assigned a value of 5 for analysis of geometric mean titer (GMT).

Neutralization assay (NT) for 2009 pH1N1

To detect Abs that could inhibit infection of cells with influenza virus, microneutralization assays were performed using MDCK cells. Samples were heat-inactivated and serial dilutions preincubated with 2009 H1N1 (A/California/04/2009; 100 $TCID_{50}$) in 96 well plates. After 1- to 2-h incubation at 37˚C in a 5% CO_2, the mixtures were added to a preformed monolayer of MDCK cells, and the plates were incubated for another 18 h. MDCK monolayers were then washed with PBS and fixed in cold 80% acetone for 1 h. The presence of viral protein was detected by ELISA using a mAb to the influenza A NP. The second Ab conjugated with peroxidase was added and incubated for another 1 h. Plates were washed and specific enzyme substrate added. The reactions were stopped with 1 N sulfuric

acid. The absorbance (A) was measured at 450 and 620 nm. The average of difference A450 and A620 was determined for duplicate wells of virus-infected (VC) and -uninfected (CC) control wells, and a neutralizing endpoint was determined by using a 50% specific signal calculation. The endpoint titer was expressed as the reciprocal of the highest dilution of serum with A450 value less than X, where X = [(average [A450-A620] of VC wells) − (average [A450-A620] of CC wells)]/2 + (average [A450-A629] of CC wells). Sera, which tested negative at a dilution of 1:20, were assigned a titer of 10 for analysis of GMT.

ELISPOT assay for spontaneous ASCs

Spontaneous Ab=secreting cells specific to influenza HA were assessed by ELISPOT assay. Briefly, ELISPOT plates (Millipore, Billerica, MA) were coated overnight at 4˚C with recombinant influenza HA (0.5 μg/well) and then blocked by incubation with 2% boiled casein in PBS for 1–2 h at 37˚C. Plates were washed (three times) and mononuclear immune cells from different tissue compartments (150,000–300,000 cells/200 μl complete medium/well) were added into Ag=coated wells in triplicate. After overnight culture at 37˚C, plates were washed (six times), followed by incubation with anti-monkey IgG conjugated with biotin (Alpha Diagnostic International, San Antonio, TX) and incubated for 2 h at 37˚C. Plate were washed (six times) and incubated with streptavidin–alkaline phosphatase (Sigma-Aldrich) for 1 h and developed by 5-bromo-4-chloro-3-indolyl phosphate-NBT-blue system (Sigma-Aldrich). The reaction was stopped by washing with tap water and air-dried. Spot-forming cells (SFC) were quantified by automated plate reader (Cellular Technology Ltd.). Keyhole limpet hemocyanin (KLH; Sigma-Aldrich) was used as a negative control Ag. The frequency of HA-specific spontaneous ASCs was defined as the numbers of SFC in HA-coated well subtracted from SFC in KLH-coated well and data are presented as SFC per million input cells.

ELISPOT assay for memory B cells

Mononuclear immune cells from different tissue compartments (1×10^6 cells/1 ml/well) were cultured in 48-well plates for 6 d in the presence of R848 (2.5 μg/ml) (InvivoGen, San Diego, CA) and IL-2 (1000 U/ml) (R&D Systems, Minneapolis, MN). Half of culture medium was replaced with fresh medium containing R848 and IL-2 at day 3. This stimulation method has been found to be optimal for assessment memory B cell response in rhesus monkeys. To enumerate HA-specific ASCs, stimulated cells (150,000–300,000 cells/200 μl complete medium/well) were added into ELISPOT plates and then processed as described for spontaneous Ab secreting cells above. The frequency of HA-specific memory B cells was defined as the numbers of spots in HA-coated well subtracted from spots in KLH-coated well and data are presented as SFC per million input cells. The samples were scored positive for HA-specific memory B cells if the ASCs were greater than the mean of pre-existing ASCs in nonstimulated cells plus 2 SDs.

Intracellular cytokine staining

T cell responses were assessed by intracellular cytokine staining. Cryopreserved PBMC and mononuclear immune cells (1×10^6 cells in 200 μl complete medium) from different tissue compartments were stimulated with NP peptide derived from A/California/04/2009 (H1N1) (122 15-mer peptide overlapping by 11 aa) at a final concentration of each peptide of 1μg/ml. All stimulated cell cultures contained 1 μg/ml anti-CD28 (clone L293; BD Biosciences) and 1 μg/ml of anti-CD49 (clone L25; BD Biosciences). Staphylococcal enterotoxin (4 μg/ml) and medium were used as positive and negative controls, respectively. After 2 h of stimulation, GolgiPlug was added to inhibit cytokine secretion, and the cell cultures were further incubated overnight. Then cells were washed and stained with a panel of Abs specific for surface markers, including anti-CD4 (clone L200; BD Biosciences), anti-CD8 (clone SK1; BD Biosciences), anti-CD95 (clone DX2; BioLegend, San Diego CA), anti-CCR7 (clone 150503; R&D Systems), anti-CD103 (clone B-Ly7; eBioscience, San Diego CA), anti–programmed death-1 (PD-1) (clone EH12.2H7; BioLegend), anti-CXCR3 (clone 1C6/CXCR3; BioLegend), and anti-CCR5 (clone 3A9; BioLegend). The stained cells were fixed/permeabilized and intracellular cytokines were stained with mAbs against IFN-γ (clone B 27; BioLegend) and IL-2 (clone MQ1-17H12; BioLegend). Finally, stained cells were analyzed by six-color flow cytometry (BDFACSCanto; BD Biosciences). The samples considered positive were those in which the percentage of cytokine-staining cells was at least twice that for the background or in which there was a distinct population of bright cytokine-positive cells.

To evaluate the role of CD69-positive memory T cells in cytokine responses, lung mononuclear immune cells were stained with anti-CD69 (clone FN50; BD Biosciences) and CD69-positive cells were depleted by fluorescence-activated cell sorter (FACSAria III; BD Biosciences). CD69-depleted lung mononuclear immune cells were stimulated with NP peptide pool and assessed for IFN-γ and IL-2 production compared with the nondepleted population.

Statistical analysis

The data were analyzed using SPSS 12.0 for Windows (SPSS, Chicago, IL). Ab titers were log transformed before testing of differences. Differences in T cell, memory B cell, and plasma cell responses were analyzed by using the Wilcoxon signed rank test and the Mann–Whitney rank sum test. $p < 0.05$ was considered statistically significant.

Results

2009 pH1N1 infection in rhesus monkeys

Of 20 monkeys seronegative for serum HAI against 2009 pH1N1, 16 received pulmonary inoculation of 2009 pH1N1 virus (2×10^6 $TCID_{50}$) at days 0 and 28, whereas 4 monkeys were inoculated with control L-15 medium (Fig. 1). Infection with 2009 pH1N1 in rhesus monkeys induced no clinical signs of disease with no difference in animal activity, body weight loss, or respiratory parameters compared with control animals (data not shown). Viral replication was detected in nasal secretions from 12 of the 16 monkeys (Fig. 2A). Although peak of virus titer was found on day 2, this could be left over inoculum. However, virus titer on days 4 and 7 in nasal wash demonstrated the virus can replicate efficiently in upper respiratory tract of rhesus monkeys. Viral replication in nasal secretions was absent after the second inoculation, wheras concomitant increases in nasal HA-specific IgG and IgA was detected (Fig. 2B), suggesting that these Ab responses may suppress local viral replication. One animal died during the course of the study at 23 d after the second virus inoculation. A diagnostic necropsy was conducted, and the cause of death was determined to be because of tuberculosis.

Serum Ab and peripheral blood T cell responses after 2009 pH1N1 infection

We measured serum Ab and peripheral blood T cell responses specific to 2009 pH1N1 virus before and after virus inoculation. Using HAI and NT assays, we showed that all 16 test animals developed serum Ab responses following infection. HAI titers (GMT = 87; 95% confidence interval [CI] = 54–142) and NT titers (GMT = 50; 95% CI = 31–80) were detected at day 14 after the 1st inoculation, continued to increase and peaked at 13 d after the 2nd inoculation with GMT HAI titers of 1,522; 95% CI = 1052–2202 and GMT NT titers of 1733; 95% CI = 1132–2653 (Fig. 3A, 3B). By day 74, the HAI and NT titers declined ∼50%. Kinetics and magnitude of peripheral blood $CD4^+$ and $CD8^+$ T cell response to NP are shown in Fig. 3C and 3D. The magnitude of the NP-specific $CD4^+$ or $CD8^+$ T cells response was defined as the combined frequency of IFN-γ-, IL-2-, and double-cytokine producing IFN-γ-plus-IL-2 cells within the $CD4^+$ or $CD8^+$ T cell

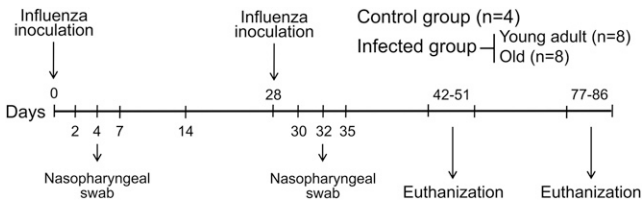

FIGURE 1. Study design for influenza inoculation and tissue collection. Rhesus monkeys were exposed via intranasal and intratracheal inoculation to 2009 pH1N1 virus at days 0 and 28. Peripheral blood, mediastinal lymph nodes, lung, spleen, and bone marrow were collected at days 14–23 and 49–58 after second virus inoculation and assessed for immune responses.

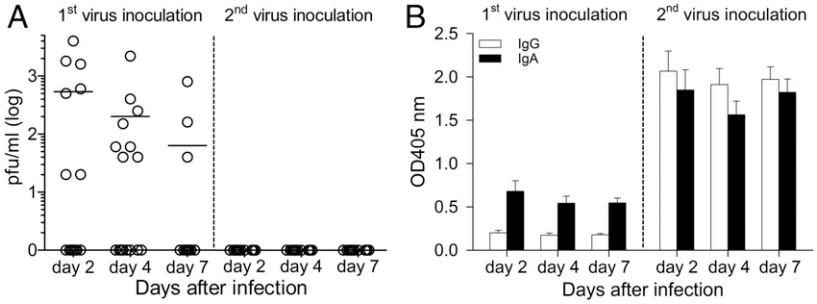

FIGURE 2. Replication of 2009 pH1N1 in rhesus monkeys and the presence of HA-specific IgG and IgA in nasal secretions. (**A**) Virus replication in nasal secretions of 16 infected animals was measured by plaque assay. Each data point represents the value for an individual monkey and horizontal lines are means. (**B**) ELISA was used to measure HA-specific IgG and IgA in nasal secretions (1:4 dilution) and expressed as OD at 405 nm. Data shown are mean ± SE.

population. Baseline responses were very low; NP-specific cytokine production by $CD4^+$ and $CD8^+$ T cells was observed 14 d after the first virus inoculation and peaked after the second inoculation (mean frequency of cytokine-producing $CD4^+$ T cells ± SE = 0.28 ± 0.04% and $CD8^+$ T cells = 0.46 ± 0.09%). Negligible influenza-specific Ab and T cell responses were detected in the control animal group.

Tissue distribution of influenza-specific memory T cells in rhesus monkeys

We next measured and compared the magnitude of memory T cell responses in different tissue compartments. The frequency of NP-specific memory T cells in peripheral blood, lung-draining mediastinal lymph nodes, lung, spleen, and bone marrow were evaluated at two time points. Half of the animals ($n = 8$) were euthanized between days 14 and 23 after the second virus inoculation when peak immune response was expected (34). This time period was termed "expansion phase." The other half were euthanized between days 49 and 58 after the second virus inoculation, when a stable distribution of memory immune cells was expected (34), termed the "contraction phase." Fig. 4A shows that during the expansion phase (days 14–23), a significantly higher frequency of NP-specific memory T cells were observed in the mediastinal lymph nodes (total frequency of 1.9%; $CD4^+$ T cells = 0.8%, $CD8^+$ T cells = 1.1%) compared with other tissues ($p < 0.05$): lung (total frequency of 0.7%; $CD4^+$ T cells = 0.4%, $CD8^+$ T cells = 0.3%), blood (total frequency of 0.7%; $CD4^+$ T cells = 0.3%, $CD8^+$ T cells = 0.4%), spleen (total frequency of 0.6%; $CD4^+$ T cells = 0.4%, $CD8^+$ T cells = 0.2%), and bone marrow (total frequency of 0.29%; $CD4^+$ T cells = 0.25%, $CD8^+$ T cells = 0.04%).

Although the T cell response during the contraction phase was still evident, a 0.4- to 2-fold decline in NP-specific memory T cell frequency was observed in the mediastinal lymph nodes, blood, and spleen compared with the expansion phase. In contrast, a 3.3-fold increase in memory T cell frequency was detected in the lung (Fig. 4B). During the contraction phase, the frequency of NP-specific memory T cells in the lung was significantly higher than that in the mediastinal lymph nodes ($p < 0.01$, total frequency of 2.4 versus 1.2%). Only a minimal number of NP-specific memory T cells (range 0.2–0.4%) were detected in blood, spleen, and bone marrow. There were not significant differences in the ratio of NP-specific memory $CD4^+$ to $CD8^+$ T cells in each tissue sample at the time points studied.

We then next evaluated the cytokine profiles of influenza-specific memory T cell responses in the lung, mediastinal lymph nodes, and peripheral blood at both time points; the expansion phase (Fig. 4C) and contraction phase (Fig. 4D). In the lung, a large proportion of NP-specific memory $CD4^+$ and $CD8^+$ T cells produced IFN-γ alone (70–77%) during the expansion phase, whereas at the contraction phase, NP-specific memory $CD4^+$ and $CD8^+$ T cells produced either IFN-γ alone (51–65%) or IFN-γ plus IL-2 (25–45%). In the mediastinal lymph nodes, we detected, at both time points, NP-specific memory $CD4^+$ T cells producing either IL-2 alone (37–50%) or IFN-γ alone (34–38%), whereas most NP-specific memory $CD8^+$ T cells produced IFN-γ alone (71–78%). In peripheral blood, about equal proportions of NP-specific

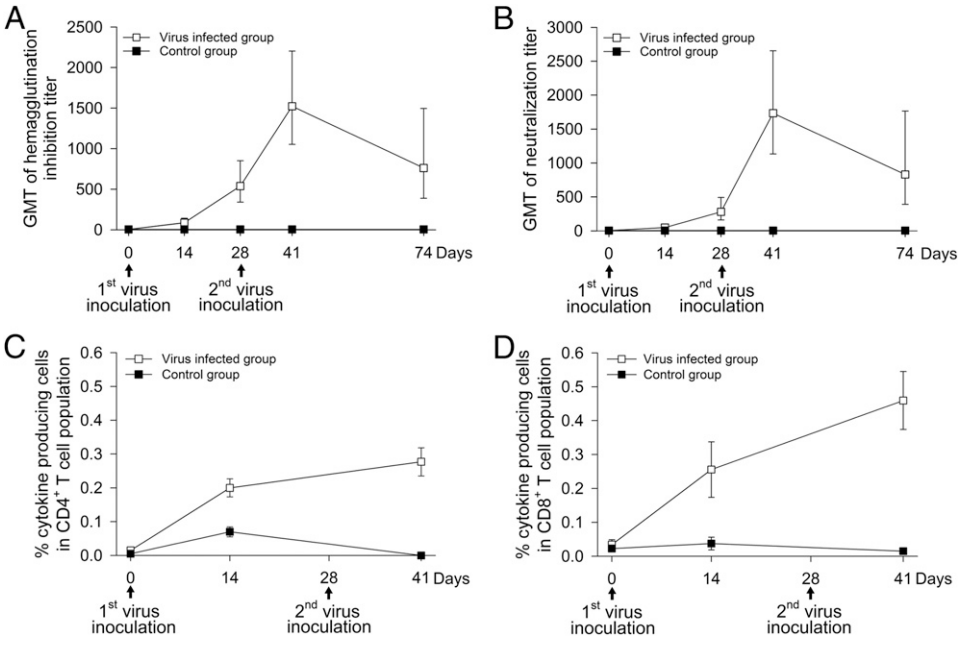

FIGURE 3. Kinetics of serum Ab and peripheral blood T cell responses. Ab responses were measured by HAI (**A**) and NT (**B**) assays and each data point represents the GMT ± SE. Peripheral blood $CD4^+$ (**C**) and $CD8^+$ (**D**) T cell responses were assessed by in vitro T cell recall assay against influenza NP. Intracellular cytokine staining was used to assess the frequencies of NP-specific cytokine-secreting T cells. Stained cells were analyzed by multicolor flow cytometry. Data shown are means ± SE of percent cytokine-producing cells (IFN-γ-, IL-2-, and IFN-γ plus IL-2) in $CD4^+$ or $CD8^+$ T cell population.

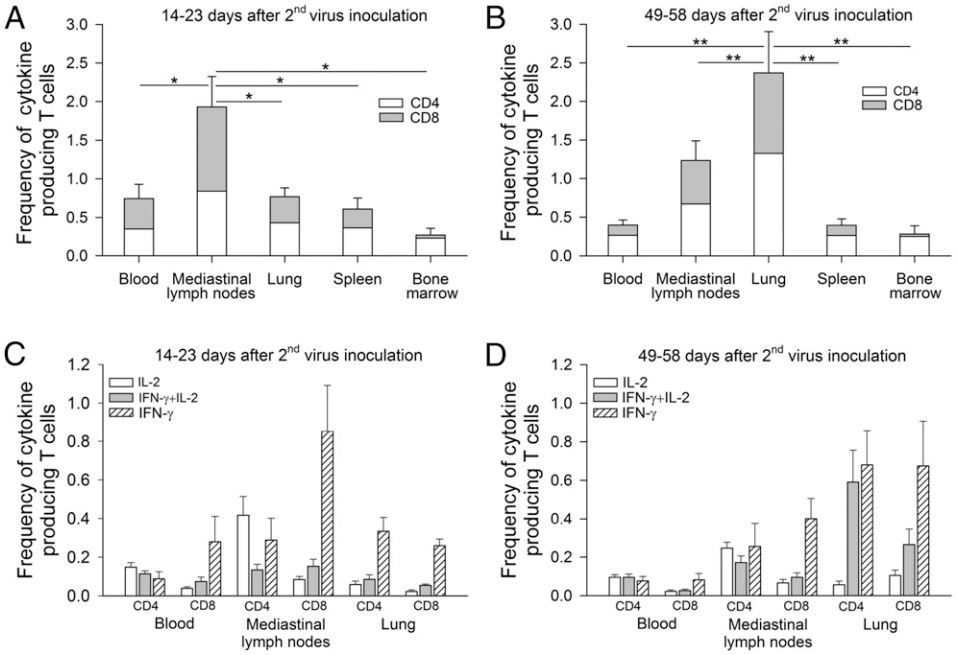

FIGURE 4. Generation of NP-specific memory $CD4^+$ plus $CD8^+$ T cells in different tissue compartments (peripheral blood, mediastinal lymph nodes, lung, spleen, and bone marrow) at the expansion phase (14–23 d after the second virus inoculation) (**A**) and the contraction phase (49–58 d after the second virus inoculation) (**B**). Data shown are means ± SE (*$p < 0.05$, **$p < 0.01$, Wilcoxon test). Cytokine profiles of NP-specific memory $CD4^+$ and $CD8^+$ T cells at the expansion phase (**C**) and contraction phase (**D**) were also evaluated by separating cells into three distinct populations based on the production of IL-2 alone, IFN-γ alone, and IFN-γ plus IL-2. Data shown are means ± SE.

memory $CD4^+$ T cells producing either IFN-γ alone (28–28%), IL-2 alone (35–42%), or IFN-γ plus IL-2 (33–35%) were observed at both time points, whereas most NP-specific memory $CD8^+$ T cells produced IFN-γ alone (63–71%).

Rhesus lung NP-specific memory T cells expressed phenotypic markers of T_{RM} cells

To investigate NP-specific memory T cells found in the lung at the contraction phase characterized as T_{RM} cells, we measured the expression of CD103 ($\alpha_E\beta_7$ integrin) and CD69 (C-type lectin), both of which are cell surface signature markers of T_{RM} cells involved in cell adhesion and tissue retention (10). Flow cytometry analysis (Fig. 5A) showed that ~40% of lung NP-specific memory $CD8^+$ T cells that produced cytokines (IFN-γ and IL-2) expressed CD103. Negligible numbers of CD103 positive NP-specific memory $CD8^+$ T cells were observed in the mediastinal lymph nodes and peripheral blood, suggesting that NP-specific memory $CD8^+$ T cells established residency only in the lung. In contrast, only a minimal proportion of lung NP-specific memory $CD4^+$ T cells (<10%) expressed CD103 (Fig. 5B).

Unlike CD103 expression, direct assessment of CD69 on NP-specific memory T cells from lung tissue was not feasible since in vitro stimulation with NP peptide pools led to CD69 expression on all NP-specific memory T cells (data not shown). We therefore first depleted CD69-positive T cells and then CD69-depleted lung mononuclear immune cells were stimulated with the NP peptide pool. In this case, the percent reduction of cytokine producing cells in a recall T cell assay of a depleted population compared with a nondepleted population would correspond to the proportion of lung NP-specific memory T cells expressing CD69. As depicted in Fig. 5C and 5D, depletion of CD69 cells resulted in a reduction of cytokine-producing cells to 37% for $CD8^+$ T cells and 43% for $CD4^+$ T cells. These data imply that 63 and 57% of lung NP-specific memory $CD8^+$ and $CD4^+$ T cells, respectively, expressed CD69. Taken together, these data suggest that following influenza infection, a substantial proportion of NP-specific memory T cells in the lung of rhesus monkeys expressed phenotypic markers of T_{RM} cells, CD103 and CD69.

Because CD103 was mainly expressed on lung memory $CD8^+$ T cells, we then compared cytokine response between lung NP-specific $CD103^+CD8^+$ and $CD103^-CD8^+$ T cells. After in vitro stimulation with the NP peptide pool, we observed no significant difference in the frequency of IFN-γ or IL-2 produced by $CD103^+CD8^+$ T cells versus $CD103^-CD8^+$ T cells (p range 0.46 – 0.74) (Fig. 5E). As PD-1, a cell surface marker of activated T cells is known to express on T_{RM} cells (16, 35), we showed that high expression of PD-1 was consistently detected on lung $CD103^+$ compared with $CD103^-$IFN-γ^+CD8^+ T cells. In addition, the expression levels of chemokine receptors involved in T cell migration into the lung (36, 37), CXCR3 and CCR5, were minimal with no significant difference between either subset (Fig. 5F).

Tissue distribution of spontaneous ASCs and memory B cells

We also assessed for the presence of spontaneous ASCs and memory B cells at the contraction phase. The frequency of spontaneous ASCs and memory B cells specific to HA was different in each studied tissue sample. As shown in Fig. 6A, unlike NP-specific memory T cells, a significantly higher frequency of HA-specific spontaneous ASCs was detected in the mediastinal lymph nodes (104 SFC/10^6 mononuclear immune cells) compared with other tissue compartments ($p < 0.01$). Fewer numbers of spontaneous ASCs were detected in the bone marrow (20 SFC/10^6 mononuclear immune cells), lung (13 SFC/10^6 mononuclear immune cells), and spleen (7 SFC/10^6 mononuclear immune cells). HA-specific spontaneous ASCs could not be detected in peripheral blood (below the established limit of detection for the assay).

For memory B cell response, a polyclonal stimulation with R848 (TLR7/8 ligand) and IL-2 were used. This type of stimulation was found to be optimal for differentiation rhesus monkey memory B cells into ASCs, without losing cell viability (Supplemental Fig. 1A, 1B). After 6 d of in vitro activation in cell culture medium, significantly higher frequency of HA-specific IgG memory B cells was detected in the mediastinal lymph nodes (325 SFC/10^6 mononuclear immune cells) compared with other tissue compartments ($p < 0.01$) (Fig. 6B). The frequency of HA-specific IgG memory B cells was low in spleen (99 SFC/10^6 mononuclear immune cells), peripheral blood (64 SFC/10^6 mononuclear immune cells), lung (20 SFC/10^6 mononuclear immune cells), and negligible in bone marrow.

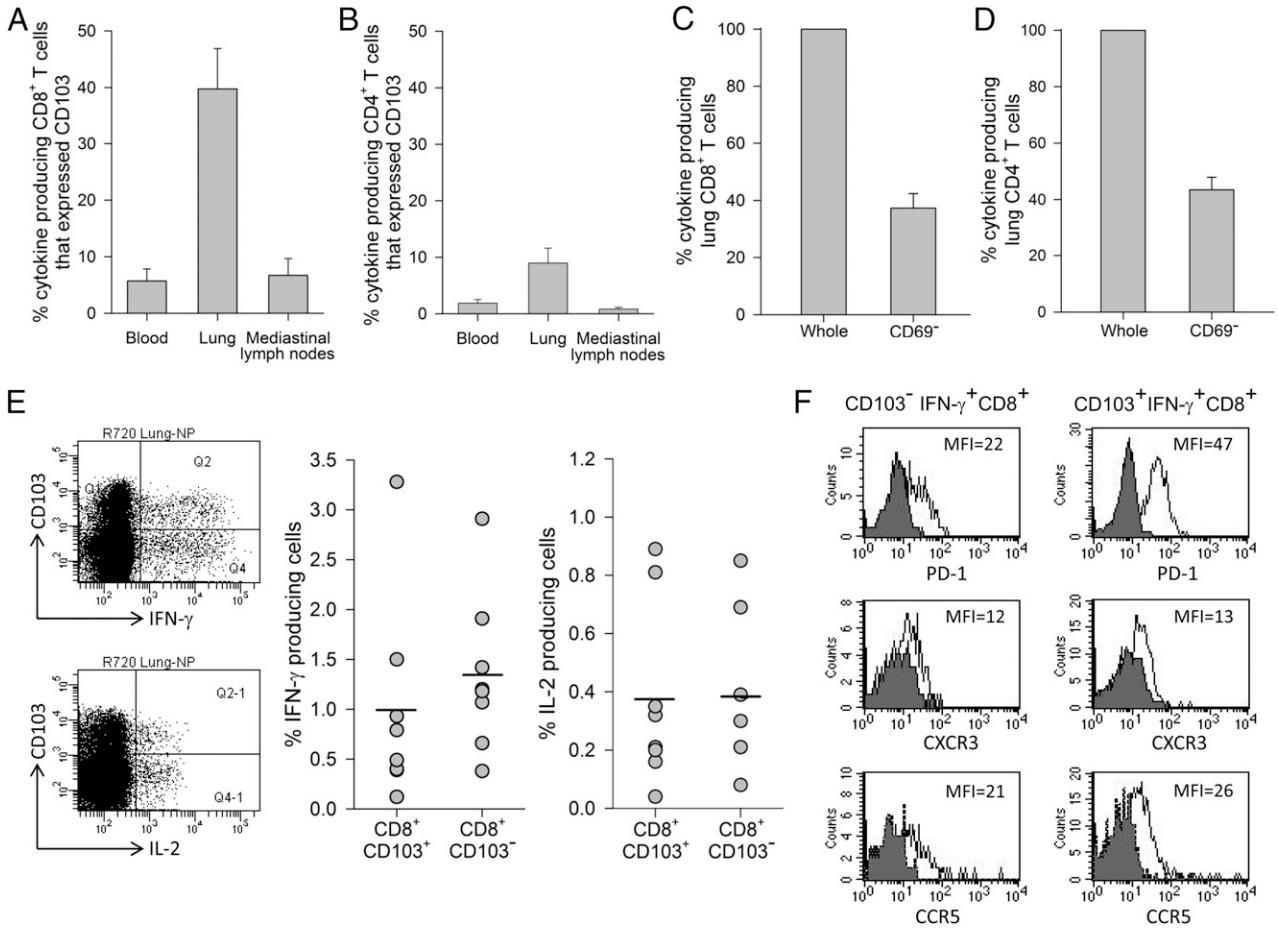

FIGURE 5. Expression of CD103 and CD69, phenotypic markers of T_{RM} on NP-specific lung memory T cells at the contraction phase. Percentage of cytokine producing $CD8^+$ (**A**) or $CD4^+$ (**B**) memory T cells that expressed CD103 in peripheral blood, lung, and mediastinal lymph nodes. Depletion of CD69-positive cells from lung mononuclear immune cells and cytokine response in NP-specific $CD8^+$ (**C**) or $CD4^+$ (**D**) memory T cells. Means ± SE of $n = 8$ are shown. Representative flow cytometry plot of lung $CD103^+$ and $CD103^-CD8^+$ T cells expressing IFN-γ and IL-2 and frequencies of IFN-γ and IL-2 production from lung $CD103^+$ and $CD103^-CD8^+$ T cells from eight animals (**E**). Each data point represents the value for an individual monkey and horizontal lines are means. Phenotype of IFN-γ–producing $CD8^+$ T cells in the lung (**F**). Shaded histograms represent isotype control and solid lines represent lung $CD103^-$ or $CD103^+IFN\gamma^+CD8^+$ T cells expressing PD-1, CXCR3, and CCR5. Data are representative of four monkeys.

KLH was used as a control Ag for coating plates. We consistently observed a negligible number of spots in Ag control KLH coated wells, suggesting the assay was specific for detecting HA. Due to a limited number of cells, we could not perform the assay to determine the number of spontaneous Ab secreting cells and memory B cells that secrete HA-specific IgA.

Influenza-specific systemic and local immune responses in young adult and old monkeys

To study age-related changes, we compared serum Ab, T cell responses in peripheral blood and local memory T, B, and spontaneous ASCs in the lung, and mediastinal lymph nodes between young adult and old monkeys. Following 2009 pH1N1 infection, all animals, regardless of age, developed immune responses against influenza. No significant difference in the magnitude and kinetics of serum Ab (HAI and NT) responses in young adult monkeys ($n = 8$; mean age ± SE = 6.5 ± 0.2 y) versus old monkeys ($n = 8$; mean age ± SE = 20.4 + 1.2 y) (Supplemental Fig. 2A). Comparable peripheral blood T cell responses between young adult and old monkeys were observed with the exception at 14 d after first virus inoculation at which the cytokine response was higher in young adult animals.

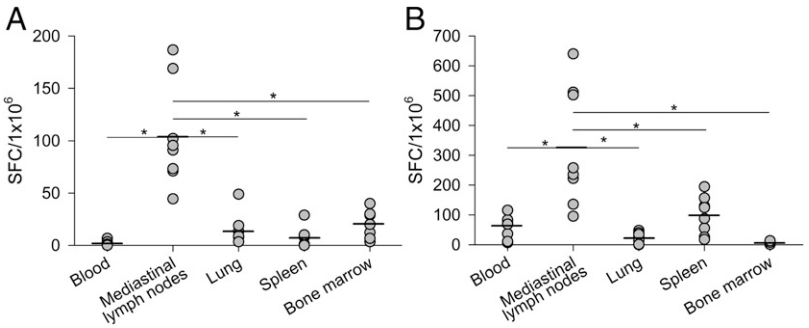

FIGURE 6. Generation of HA-specific spontaneous ASCs and memory B cells at the contraction phase. Mononuclear immune cells isolated from different tissue compartments were assayed for the presence of spontaneous ASCs (**A**) and memory B cells (**B**) specific to influenza HA using ELISPOT assay. Each data point represents value for an individual monkey and horizontal lines are means (*$p < 0.01$, Wilcoxon test).

There were not significant differences in the magnitude of cytokine-producing lung memory T cells, CD103 expression on cytokine-producing lung memory $CD8^+$ T cells, mediastinal lymph node spontaneous ASCs, and memory B cells in young adult monkeys ($n = 4$; mean age $\pm$ SE = 6.5 $\pm$ 0.3) and old monkeys ($n = 4$; mean age $\pm$ SE = 20.5 $\pm$ 2.5) at the contraction phase (Supplemental Fig. 2B).

Discussion

One of the key challenges for vaccinologists is to develop vaccines that induce site-specific protective immunity to attempt to mimic the immune response to natural infection. The generation of T_{RM} cells and memory B cells as part of the local lung immune response against influenza infection has recently been demonstrated in a mouse model of influenza infection (15, 22, 23, 35). To further explore the role of local immunity, we used a nonhuman primate model of influenza infection to analyze the tissue distribution of memory T and B cells. Our findings are consistent with previous reports that pulmonary infection of rhesus monkeys with 2009 pH1N1 induces no clinical disease associated with limited viral replication (32, 33) and that rhesus monkeys pulmonary infected with 2009 pH1N1 developed both systemic and local immune responses. Influenza-specific adaptive immune cells including memory T, B, and spontaneous ASCs were detected in several tissue compartments but differed in tissue tropisms.

During the expansion phase 14–23 d after second virus inoculation, the proportion of NP-specific memory T cells in the lung-draining mediastinal lymph nodes was significantly higher than in the lung. These finding may not be surprising because priming of naive T cells by dendritic APCs occurs in the mediastinal lymph nodes (38, 39). Preferential localization of memory T cells to the lungs occurred during the contraction phase, 49–58 d after second virus inoculation. These results agree with previous mouse data, which suggest that the tissue tropism of the natural infection influences the localization of memory T cells (40). The markedly increased lung NP-specific memory T cells and the decrease of mediastinal lymph node memory T cells at the later contraction phase likely reflect the migration of memory T cells from the lymph nodes to the lung. Despite similarity in the magnitude of NP-specific memory T cell responses in peripheral blood and in the lung at the expansion phase, the frequency of NP-specific memory T cells in the lung at the contraction phase was 6-fold greater than that in peripheral blood. In addition, the number of polyfunctional memory $CD4^+$ and $CD8^+$ T cells producing double cytokines, IFN-γ plus IL-2, in the lung was 6- to 10-fold greater in frequency than that in peripheral blood, suggesting a possible functional difference between memory T cells from the two compartments. Increase in the number of polyfunctional T cells that produce multiple cytokines is known to associate with protective immunity in many infection models (41–44).

T_{RM} cells coexpressing CD103 and CD69 have been identified in mice and recently in humans (11, 45, 46). The $\alpha_E\beta_7$ integrin CD103 promotes cell adherence to E-cadherin expressed on tissue epithelial cells (47), whereas C-type lectin CD69 inhibits sphingosine-1-phosphate receptor 1, leading to tissue retention (48, 49). In this study, we identified influenza-specific lung memory T cells expressing CD103 and CD69 in rhesus monkeys. Consistent with mouse data, CD103 expression was detected mainly on $CD8^+$ memory T cells but negligible on $CD4^+$ memory T cells, whereas CD69 expression was detected on both memory $CD8^+$ and $CD4^+$ T cells (11, 45). Recent data show a differentiation pathway from $CD69^+CD103^-$ to $CD69^+CD103^+$ T_{RM} cells and suggest a delay of CD103 expression compared with CD69 expression (50). These findings may explain why the proportion of lung CD103-positive NP-specific $CD8^+$ T cells observed in this study was consistently less than CD69-positive NP-specific $CD8^+$ T cells (40 versus 63%). A limitation of our study is that we did not sample far out enough in time to assess whether CD103 would increase in number. Similar to influenza-infected mice (51), influenza infection in rhesus monkeys generates two populations of lung memory T cells, ones expressing T_{RM} cell phenotypic markers CD103 and CD69, which are involved in cell adhesion and tissue retention and likely localize in lung parenchyma, whereas the others lack CD69 and CD103 expression and thus likely localize in lung vasculature. Unlike mice in which the anatomical location of vascular and lung tissue memory T cells can be differentiated by intravascular Ab staining combined with tetramer labeling (51, 52), there are significant technical restrictions to using such a technique in rhesus monkeys.

It has been suggested that the ability of memory T cells to home to the lung parenchyma as T_{RM} is critical to control lung pathogens such as influenza and *Mycobacterium tuberculosis* (15, 16). Strategically well-positioned in the lung tissue, T_{RM} cells could rapidly recognize, mediate cytotoxicity and secrete cytokines to control local microbe-infected cells. Our study on cytokine profiles of NP-specific lung memory T cells at the contraction phase indicates that a large proportion of both lung $CD8^+$ and $CD4^+$ memory T cells produced IFN-γ alone or IFN-γ plus IL-2. Further detailed analysis suggests that there is no different in the levels of IFN-γ and IL-2 responses between lung $CD103^+CD8^+$ versus $CD103^-CD8^+$ memory T cells. Recent observations suggest that airway $CD8^+$ T_{RM} cells produce IFN-γ faster than systemic memory $CD8^+$ T cells and are responsible for rapid protection against respiratory infection (53). In this study, we did not compare the kinetics of cytokine production between lung $CD103^+$ $CD8^+$ versus $CD103^-CD8^+$ memory T cells. The ability to secrete IFN-γ and IL-2 is crucial for local lung immunity. T_{RM} cell–derived IFN-γ is known to be critical for recruitment of circulating memory $CD8^+$ T and B cells to the site of infection via an IFN-γ–induced VCAM-1 pathway (54) and is responsible for the induction of broad antiviral protein; the IFN-induced transmembrane protein 3 in local tissue (55). Mouse lung T_{RM} cells that lack IFN-induced transmembrane protein 3 are more susceptible to influenza infection than their normal counterparts (56). IL-2 produced by T_{RM} cells could rapidly upregulate granzyme B on innate NK cells involved in direct killing of infected cells (54). The ability of rhesus lung-resident memory T cells to recognize a conserved influenza NP suggests that they may play a role in heterosubtypic immunity against emerging influenza virus variants.

Our findings that lung $CD103^+IFN\text{-}\gamma^+CD8^+$ T cells express higher levels of PD-1 than $CD103^-IFN\text{-}\gamma^+CD8^+$ T cells seems to support previous observations in mice that influenza-specific lung T_{RM} express higher PD-1 than non-T_{RM} cells (35). Alternatively, the observed expression of PD-1 may result from an in vitro stimulation with NP peptide pool (57), and this may suggest a high activation status of $CD103^+IFN\text{-}\gamma^+CD8^+$ T cells. In any event, more work is needed to determine regulatory role of PD-1 expression of T_{RM} cells. In addition, we observed only minimal expression of CXCR3 and CCR5, on both $CD103^+$ and $CD103^-$ $IFN\text{-}\gamma^+CD8^+$ T cell subsets. These findings differ from recent observations in *M. tuberculosis*–infected mice, which show high expression of CXCR3 on lung T_{RM} cells (16). More study of chemokine receptors on influenza-specific lung T_{RM} cells is required to elucidate to importance of chemokine receptors in establishing tissue residence.

Plasma cells and memory B cells are components of humoral immunity crucial for protection against influenza infection. We

observed a high frequency of long-lived HA-specific spontaneous ASCs and memory B cells in the mediastinal lymph nodes and only low frequencies in the lung of rhesus monkeys. The findings differ from recent mouse studies in which large numbers of influenza-specific ASCs and memory B cells are detected in the lung after influenza infection (22, 23, 58). These mouse ASCs and memory B cell are generated during germinal center activation within ectopic lymphoid-like structure in the lung known as induced BALT (iBALT) (59, 60). Development of iBALT in murine lung is commonly observed and persists for a long time after influenza infection (61). We did not investigate the formation of iBALT in the lung of influenza-infected monkeys. However, infection of rhesus monkeys with 2009 pH1N1in our study was self-limited and asymptomatic with no clinical signs of intense lung inflammation, conditions that do not favor iBALT formation (62, 63).

Accumulating data suggest that humoral and cellular immune responses are impaired in aged individuals, leading to the increased susceptibility to influenza infection and decreased immune response to influenza vaccine (64). Our findings show that the serum Ab (HAI and NT titers) and peripheral blood T cell responses were mostly comparable between young adult and old monkeys following 2009 pH1N1infection. These findings differ from recent observations indicating that influenza vaccine-induced Ab responses are significantly reduced in old rhesus monkeys compared with young adult animals (65). We did not detect any significant difference in young adult versus old monkeys with regard to the frequency of NP-specific memory T in the lung and the proportion of lung NP-specific memory T cells expressing CD103. In addition, the frequency of spontaneous Ab secreting cells and memory B cells in the lung mediastinal lymph nodes did not correlate with the age of animals. This study was conducted using a small sample size, and the average age in the older group was 20.4 y (equivalent to a 63-y-old humans), limiting conclusions to some degree.

Our findings from a rhesus monkey model of 2009 pH1N1 infection support the current concept that the site of pathogen infection determines the localization of immune memory cells. Following influenza infection, we detected preferential localization of memory T and B cells in the lung and lung-draining mediastinal lymph nodes, respectively. We confirm recent observations that immune responses in the lung differ from those in peripheral blood, which are commonly used as an indicator of memory response following infection or vaccination. Analysis of immune responses in specific tissue compartment where protection is needed against localized infection such as influenza, tuberculosis, and liver-stage malaria provides important insights into protective immunity and will help guide vaccine development.

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Disclosures

The authors have no financial conflicts of interest.

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