ซีดี 103^{+} ทิชชูเรสซิเดนต์เม็มโมรีทีเซลล์ในโรคไลเคนแพลนัสช่องปาก

นายพิสุทธิ์ อำนวยพาณิชย์

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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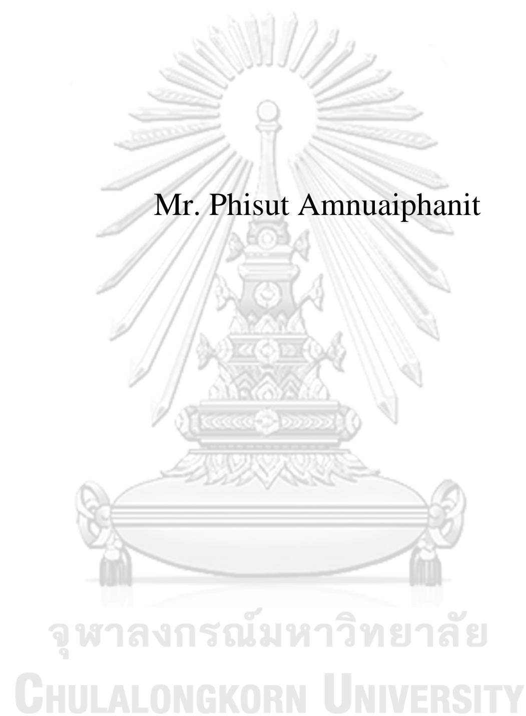
วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเวชศาสตร์ช่องปาก ภาควิชาเวชศาสตร์ช่องปาก
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CD103^{+} TISSUE RESIDENT MEMORY T CELLS IN ORAL LICHEN PLANUS

Mr. Phisut Amnuaiphanit



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Oral Medicine
Department of Oral Medicine
Faculty of Dentistry
Chulalongkorn University
Academic Year 2017
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Thesis Title	$CD103^{+}$ TISSUE RESIDENT MEMORY T CELLS IN ORAL LICHEN PLANUS
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พิสุทธิ์ อำนวยพาณิชย์ : ซีดี 103^+ ทิชชูเรสซิเดนต์เม็มโมรีทีเซลล์ในโรคไลเคนแพลนัสช่องปาก ($CD103^+$ TISSUE RESIDENT MEMORY T CELLS IN ORAL LICHEN PLANUS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทพญ. ดร.พรพรรณ พิบูลย์รัตนกิจ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ทพ.กิตติพงษ์ ดนุไทย, หน้า.

โรคไลเคนแพลนัสช่องปาก เป็นโรคที่มีการอักเสบเรื้อรัง ซึ่งเป็นผลมาจากความผิดปกติของทีเซลล์ที่เข้ามาในรอยโรค ทิชชูเรสซิเดนต์เม็มโมรีทีเซลล์ เป็นทีเซลล์ที่มีซีดี 103 แสดงบนผิวเซลล์ เซลล์ชนิดนี้อยู่ประจำเป็นเวลานานในเยื่อเมือกช่องปากที่เคยเกิดการอักเสบมาก่อน เพื่อทำหน้าที่ป้องกันเฉพาะส่วนต่อการรุกรานของจุลชีพก่อโรคได้อย่างรวดเร็ว เซลล์ชนิดนี้ยังถูกคาดคะเนว่ามีส่วนเกี่ยวข้องกับโรคหลายโรค รวมไปถึงโรคไลเคนแพลนัสช่องปากด้วย การศึกษานี้มีวัตถุประสงค์เพื่อเปรียบเทียบจำนวนและสัดส่วนของซีดี 103^+ ทิชชูเรสซิเดนต์เม็มโมรีทีเซลล์ระหว่างเนื้อเยื่อโรคไลเคนแพลนัสช่องปาก กับเยื่อเมือกช่องปากปกติ การศึกษาทำโดยนำเนื้อเยื่อทั้ง 2 ชนิด ชนิดละ 15 ชิ้น มาย้อมด้วยแอนติบอดีต่อซีดี 3, ซีดี 4, ซีดี 8 และซีดี 103 ด้วยวิธีทางอิมมูโนฮิสโตเคมี จากนั้นจึงนำข้อมูลที่ได้มาทดสอบทางสถิติ ซึ่งประกอบด้วย Unpaired t-test, Paired t-test, Mann-Whitney U test และ Wilcoxon Signed Ranks test โดยกำหนดให้ $p < 0.05$ มีนัยสำคัญทางสถิติ ผลการศึกษาพบว่า เนื้อเยื่อโรคไลเคนแพลนัสช่องปากมีจำนวนซีดี 3^+, ซีดี 4^+, ซีดี 8^+ และซีดี 103^+ เซลล์ ต่อพื้นที่ (เซลล์/ตารางมิลลิเมตร) มากกว่าเยื่อเมือกปกติ ($p < 0.001$) โดยเซลล์เหล่านี้ถูกพบอยู่ในชั้นเนื้อเยื่อยึดต่อใต้เยื่อบุผิว มากกว่าในชั้นเยื่อบุผิว ($p < 0.005$) เนื้อเยื่อโรคไลเคนแพลนัสช่องปากยังมีร้อยละของซีดี 103^+ เซลล์ ต่อซีดี 3^+ เซลล์ ในชั้นเนื้อเยื่อยึดต่อใต้เยื่อบุผิว มากกว่าเยื่อเมือกปกติ ($p < 0.001$) แต่ไม่พบความแตกต่างกันในชั้นเยื่อบุผิวระหว่างเนื้อเยื่อทั้ง 2 ชนิด ($p = 0.062$) นอกจากนี้ยังพบว่า เนื้อเยื่อโรคไลเคนแพลนัสช่องปากมีร้อยละของซีดี 103^+ เซลล์ ต่อซีดี 3^+ เซลล์ ในชั้นเนื้อเยื่อยึดต่อใต้เยื่อบุผิว น้อยกว่าในชั้นเยื่อบุผิวด้วย ($p < 0.001$) โดยสรุป การศึกษานี้แสดงให้เห็นว่า เนื้อเยื่อโรคไลเคนแพลนัสช่องปากมีความหนาแน่นของซีดี 103^+ เซลล์ เพิ่มขึ้นจากเยื่อเมือกปกติหลายเท่า ซึ่งอาจบ่งบอกได้ว่า ซีดี 103^+ ทิชชูเรสซิเดนต์เม็มโมรีทีเซลล์ อาจมีความสัมพันธ์กับพยาธิกำเนิดของโรคไลเคนแพลนัสช่องปาก

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สาขาวิชา	เวชศาสตร์ช่องปาก	ลายมือชื่อ อ.ที่ปรึกษาหลัก	....
ปีการศึกษา	2560	ลายมือชื่อ อ.ที่ปรึกษาร่วม	....

5975832432 : MAJOR ORAL MEDICINE
KEYWORDS: CD103 / ORAL LICHEN PLANUS / TISSUE RESIDENT MEMORY T CELL / TRM CELL

PHISUT AMNUAIPHANIT: $CD103^+$ TISSUE RESIDENT MEMORY T CELLS IN ORAL LICHEN PLANUS. ADVISOR: ASSOC. PROF. PORNPAN PIBOONRATANAKIT, Ph.D., CO-ADVISOR: ASSOC. PROF. KITTIPONG DHANUTHAI, pp.

Oral lichen planus (OLP) is a chronic inflammatory disease that results from a disorder of infiltrating T cells. Tissue resident memory T cells (T_{RM} cells) are CD103-expressing T cells that persist within previously inflamed oral mucosa in the long term to provide locally rapid defensive responses against encountered pathogens. They are also speculated to be associated with some diseases including OLP. This study aimed to determine the number and the proportion of $CD103^+$ T_{RM} cells in the OLP lesions as compared to the normal mucosa. Immunohistochemical study of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells in 15 OLP tissues and 15 normal mucosa tissues was performed. An unpaired t-test, a paired t-test, a Mann-Whitney U test and a Wilcoxon Signed Ranks test were used to analyze the data. A p-value < 0.05 was considered statistically significant. The results revealed significant increases in the numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cells/mm^2) in the OLP lesions as compared to the normal mucosa ($p < 0.001$). These cells were frequently expressed in the lamina propria rather than the epithelium ($p < 0.005$). The proportion of $CD103^+$ cells to $CD3^+$ cells (%) in the lamina propria of the OLP lesions was lower than that of the normal mucosa. ($p < 0.001$), but this significant difference was not found in the epithelium between both tissues ($p = 0.062$). Furthermore, the OLP lamina propria showed lower proportion of $CD103^+$ cells to $CD3^+$ cells than the OLP epithelium ($p < 0.001$). In conclusion, this study demonstrates the several-fold increase in the density of $CD103^+$ cells in the OLP lesions that may suggest the association between $CD103^+$ T_{RM} cells and the pathogenesis of OLP.

Department:	Oral Medicine	Student's Signature ....
Field of Study:	Oral Medicine	Advisor's Signature ....
Academic Year:	2017	Co-Advisor's Signature ....

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ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to my advisor, Associate Professor Pornpan Piboonratanakit, as well as my co-advisor, Associate Professor Kittipong Dhanuthai, for their supervision and continuous support over the course of my post-graduated program. They also provided valuable advice and detailed correction that made my thesis complete.

I would like to give special thankfulness to Professor Rangsini Mahanonda and Dr. Sathit Pichyangkul who helped me draw an idea about the association of tissue resident memory T cells and oral lichen planus, and also introduced many interesting aspects of this topic. I am grateful to Dr. Paksinee Kamolratanakul for her help in collecting biopsy specimens. I also thank Assistant Professor Soranun Chantarangsu for her advice in statistical analysis.

My sincere thanks go to Professor Kobkan Thongprasom and Assistant Professor Patrayu Taebunpakul for being thesis committee members. I value and appreciate their kindness suggestions in order to improve my thesis project.

I thank Mr. Noppadol Sa-Ard-Iam for his indispensable help in immunohistochemical technique and Mr. Somchai Yodsanga for specimen preparation. My thanks are extended to staff in the Department of Oral Medicine, Faculty of Dentistry, Chulalongkorn University for their assistance.

I gratefully acknowledge the contribution of Research Unit for Immunopathological/Clinical Research in Periodontal Disease as well as Research Unit in Oral Diseases, Faculty of Dentistry, Chulalongkorn University.

This study was supported by Faculty Research Grant (DRF61015), Faculty of Dentistry, Chulalongkorn University and Graduate School Thesis Grant, Chulalongkorn University.

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LIST OF ABBREVIATIONS

1. ACE	Angiotensin-converting-enzyme
2. APC	Antigen presenting cell
3. C3	Complement 3
4. CCL	Chemokine (C-C motif) ligand
5. CCR	C-C chemokine receptor
6. CD	Cluster of differentiation
7. CTL	Cytotoxic T cell
8. DAB	3,3'-Diaminobenzidine
9. DI	Deionized (water)
10. DIF	Direct immunofluorescence
11. DNA	Deoxyribonucleic acid
12. DPBS	Dulbecco's phosphate-buffered saline
13. EBV	Epstein-Barr virus
14. EDTA	Ethylene diamine tetraacetic acid
15. FasL	Fas ligand
16. HCV	Hepatitis C virus
17. HHV	Human herpesvirus
18. HPV	Human papillomavirus
19. HRP	Horseradish peroxidase
20. IFN	Interferon
21. Ig	Immunoglobulin
22. IL	Interleukin
23. LL-37	Leucine leucine-37 or cathelicidin antimicrobial peptide-18
24. LP	Lichen planus
25. MHC	Major histocompatibility complex
26. MMP	Matrix metalloproteinase
27. NK	Natural killer (cell)
28. NSAID	Non-steroidal anti-inflammatory drug
29. OLCL	Oral lichenoid contact lesion

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30. OLDR	Oral lichenoid drug reaction
31. OLL	Oral lichenoid lesion
32. OLL-GVHD	Oral lichenoid lesion of graft-versus-host disease
33. OLP	Oral lichen planus
34. PDC	Plasmacytoid dendritic cell
35. RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
36. RNA	Ribonucleic acid
37. S1PR1	Sphingosine-1-phosphate receptor 1
38. T_{CM}	Central memory T (cell)
39. TCR	T cell receptor
40. T_{EM}	Effector memory T (cell)
41. Tfh	T follicular helper (cell)
42. TGF	Transforming growth factor
43. Th	T helper (cell)
44. TNF	Tumor necrosis factor
45. TNFR1	Tumor necrosis factor receptor 1
46. Treg	Regulatory T (cell)
47. T_{RM}	Tissue resident memory T (cell)
48. T_{SCM}	Stem cell memory T (cell)
49. TBST	Tris-buffered saline with Tween
50. VCAM1	Vascular cell adhesion molecule 1

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CHAPTER I

INTRODUCTION

Background and rationale

Oral lichen planus (OLP) is a chronic inflammatory disease that results from a disorder of T cell-mediated immune response[1]. The exact etiology of OLP remains uncertain. Numerous predisposing factors have been postulated, such as viral infections[2-4] bacterial products[5, 6] and stress[7]. A pathogenesis of OLP is traditionally explained that $CD8^+$ T cells are activated by the particular predisposing factors and they subsequently trigger basal epithelial cells to undergo apoptosis[8]. A variety of cells and cytokines, such as T helper 1 (Th1) cells, regulatory T (Treg) cells, dendritic cells, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), are assumed to be involved in this lesion[8-10].

Tissue resident memory T (T_{RM}) cells are a non-recirculating population of the memory T cells that persist in the previously infected or inflamed skin and mucosa in the long term to provide locally rapid protective functions against pathogens[11, 12]. Similar to other memory T cells, they also have a cross-reactivity feature. They are characterized by the expression of CD69 and CD103 surface molecules[13].

Although T_{RM} cells exert paramount roles in the protective functions, they can elicit tremendous destructive outcomes resulting in autoimmune diseases[14]. Some diseases have been reported to be probably related with them such as psoriasis[15], fixed drug eruption[11, 16] and OLP[17]. The hypothetic model about T_{RM} cell-mediated pathogenesis of OLP is as follows. In the previously infected or inflamed oral mucosa, cross-reactive T_{RM} cells that recognize self-antigens may directly trigger the apoptosis of the basal epithelial cells. Dead or dying epithelial cells release self-nucleic acids that may be combined with some molecules and in turn activate T_{RM} cells in a vicious cycle manner.

To support the hypothesis, this study was conducted to determine the expression of $CD103^+$ T_{RM} cells in OLP as compared to normal mucosa by means of single-labelling immunohistochemistry. Thus, this study is considered the first step to

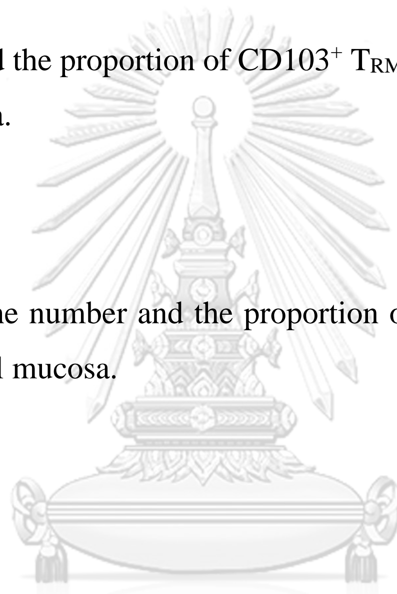
explicate the association of T_{RM} cells with the pathogenesis of OLP, which in turn will gain better understanding of this disease course.

Research question

Are the number and the proportion of $CD103^{+}$ T_{RM} cells in OLP different from those in normal mucosa?

Research hypothesis

The number and the proportion of $CD103^{+}$ T_{RM} cells in OLP are different from those in normal mucosa.



Research objective

To determine the number and the proportion of $CD103^{+}$ T_{RM} cells in OLP as compared to the normal mucosa.

Research field

Oral medicine

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Research types

Analytical and experimental research

Conceptual framework

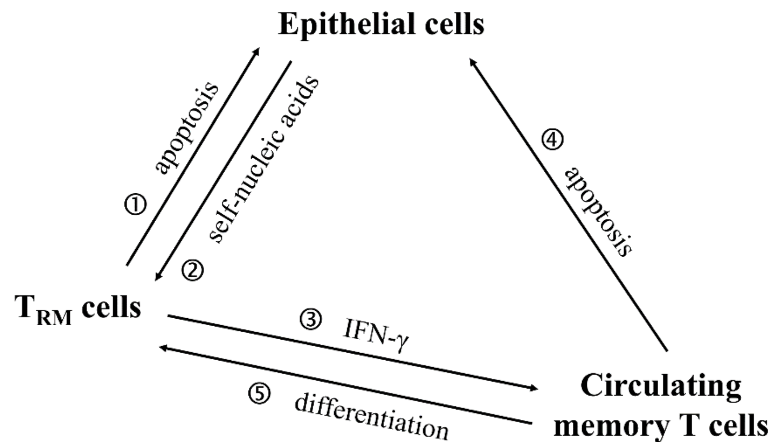

Figure 1 A conceptual framework

In the previously infected or inflamed oral mucosa, (1) cross-reactive T_{RM} cells that recognize self-antigens may directly trigger the apoptosis of the basal epithelial cells via 3 possible mechanisms comprising TNF-α/TNF-α receptor 1 (TNFR1), granzyme B with perforin and Fas (CD95)/Fas ligand (FasL or CD95L). (2) Dead or dying epithelial cells release self-RNAs and self-DNAs that may be combined with some molecules and in turn activate T_{RM} cells in a vicious cycle manner. (3) T_{RM} cells may also secrete IFN-γ to recruit both $CD4^+$ and $CD8^+$ circulating memory T cells to the inflamed area. (4) $CD8^+$ memory T cells with assistance from $CD4^+$ memory T cells are stimulated to elicit the apoptosis of the basal epithelial cells. (5) After the inflammation is suppressed, the minority of these circulating memory T cells may differentiate into T_{RM} cells for further challenges *[Figure 1]*.

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Research framework

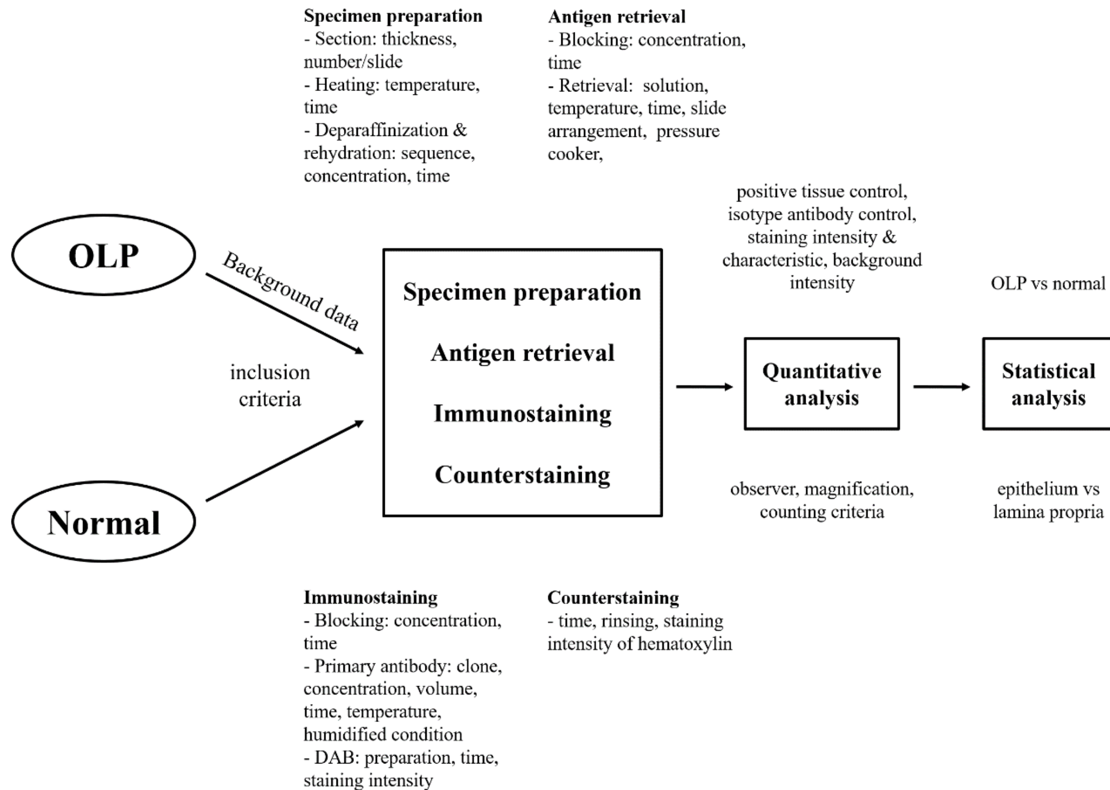

Figure 2 A research framework

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Significance of research

The results of this study will provide the profiles of $CD103^{+}$ T_{RM} cells with respect to number, proportion and anatomical localization in OLP. This research is considered the first step for further study on the association between T_{RM} cells and the immunopathogenesis of OLP, which in turn will be helpful in better understanding of this disease course and may have important consequences for the development of promising immunotherapeutic strategies for OLP.

Keywords

CD103, Oral lichen planus, Tissue resident memory T cell, T_{RM} cell

CHAPTER II

REVIEW OF LITERATURE

Oral lichen planus

Lichen planus (LP) is recognized as a chronic inflammatory disease that results from a disorder of T cell-mediated immune response[1]. The lesions can manifest at skin, mucous membrane or both[18]. LP eruption in the oral cavity is known as OLP. It affects both stratified squamous epithelium and lamina propria of the oral mucosa. The disease represents in most patients by a relapsing-remitting course[19]. OLP affects approximately 1-2% of the population in all racial groups, but markedly presents in the middle-aged females with an average age of 50.9 ± 13.1 years[18, 20]. OLP can transform to oral squamous cell carcinoma with a malignant transformation rate of 1.1%[21].

Clinical manifestations

The patients with OLP commonly complain of a burning sensation, particularly when eating hot or spicy food. Other complaints are pain, roughness, while some patients do not complain any symptom. Classical clinical features of OLP are bilateral white striations of the oral mucosa, predominantly at the buccal mucosa. Other erupted sites are gingiva, mucobuccal fold, tongue, labial mucosa, lips, hard palate, and floor of mouth. The lesions commonly emerge at more than one site. OLP has been categorized into 5 main clinical types consisting of reticular, papular, plaque-like, atrophic and ulcerative (erosive). The majority of the lesions exhibit as the atrophic type[1, 20]. Besides, there are unusual variants of OLP presenting as pigmented and bullous types[22, 23].

Histopathological features

The distinct histopathological features of OLP are described as a lymphocytic band along the superficial laminal propria and liquefactive degeneration of the basal cell layer of the epithelium. The surface epithelium may show signs of hyper-parakeratosis, hyper-orthokeratosis, acanthosis of stratum spinosum and serrated rete

ridges. Colloid bodies (Civatte bodies) may be found in the basal layer, the epithelium and the superficial part of the lamina propria[24, 25]

Direct immunofluorescence (DIF) is a supplementary test for the diagnosis of OLP. About 83% of OLP lesions demonstrates positive DIF patterns. The most common finding is the fibrinogen deposition in a shaggy pattern along a basement membrane zone with or without immunoglobulin M (IgM) deposition on colloid bodies in the upper lamina propria. Granular IgM, granular complement 3 (C3) and linear C3 deposition along the basement membrane, as well as, IgA and C3 deposition on the colloid bodies can also be found in the lesions[26].

Diagnostic criteria

Criteria using both clinical and histopathological characteristics for diagnosis of OLP was developed by World Health Organization (WHO) in 1978[24] and a modified version was developed by van der Meij and van der Waal in 2003[27] *[Table 1]*.

Table 1 Modified WHO diagnostic criteria of OLP and oral lichenoid lesion (OLL)[27]

Clinical criteria

- Presence of bilateral, more or less symmetrical lesions
- Presence of a lace-like network of slightly raised gray-white lines (reticular pattern)
- Erosive, atrophic, bullous and plaque-type lesions are only accepted as a subtype in the presence of reticular lesions elsewhere in the oral mucosa

* In all other lesions that resemble OLP but do not complete the aforementioned criteria, the term *"clinically compatible with"* should be used

Histopathological criteria

- Presence of a well-defined bandlike zone of cellular infiltration that is confined to the superficial part of the connective tissue, consisting mainly of lymphocytes
- Signs of liquefaction degeneration in the basal cell layer
- Absence of epithelial dysplasia

* When the histopathologic features are less obvious, the term *"histopathologically compatible with"* should be used

Final diagnosis OLP or OLL

To achieve a final diagnosis, clinical as well as histopathologic criteria should be included:

- OLP - A diagnosis of OLP requires fulfillment of both clinical and histopathologic criteria
- OLL - The term OLL will be used under the following conditions:
  1. Clinically typical of OLP but histopathologically only compatible with OLP
  2. Histopathologically typical of OLP but clinically only compatible with OLP

Managements

The patients with asymptomatic reticular OLP generally require no treatment but only periodic observation for changes, while the patients with symptomatic OLP are usually treated by topical corticosteroids including 0.05% clobetasol propionate in orabase, 0.1% fluocinolone acetonide in orabase, 0.1% fluocinolone acetonide solution, 0.1% triamcinolone in orabase, 0.1% triamcinolone acetonide mouthwash and 0.05% dexamethasone mouthwash[28, 29]. Intra-lesional corticosteroids and systemic corticosteroids may be indicated for the patients who do not respond to the topical forms[30]. Other treatment modalities that have been suggested to manage OLP include systemic and topical retinoids, topical cyclosporine, topical and systemic tacrolimus, topical pimecrolimus, topical thalidomide, aloe vera, curcuminoids, hyaluronic acid, lycopene, low intensity laser and psychiatric therapy[30, 31]. However, there has been insufficient evidence to support the superior effectiveness of any specific treatment for OLP[32].

Etiology

There have been controversies about the definite etiology of OLP. Numerous predisposing factors associated with OLP have been reported.

(1) Genetic background – Polymorphisms of genes encoding various cytokines particularly IFN-γ and TNF-α have been supposed to play roles in the pathogenesis of OLP[33].

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(2) Virus – Human papillomavirus (HPV), Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV-6) have been suggested to be related to OLP[2, 3]. But the study in the Thai patients indicated a low prevalence of HPV infection in OLP lesions[34]. Besides, Hepatitis C virus (HCV) has been intensely proposed to be the causing factor of OLP, nevertheless, this assumption could be demonstrated only in limited geographical areas such as Italy, Spain, Turkey and Japan[35]. The study in the Thai patients showed small prevalence of HCV infection among the patients with OLP[4].

(3) Bacteria – Inflammation of OLP may associate with oral bacteria. Fusobacteria and Campylobacter have been reported to be significantly increased in the saliva of the patients with erosive OLP. The gingival OLP lesions seemed to be

improved upon using antimicrobial mouthwash as well as removal of plaque and calculus[5, 6].

(4) Psychological factors – The earlier study revealed that stress and anxiety was associated with exacerbation of OLP. The patients with OLP often associated with increased level of stress and anxiety[7].

(5) Autoimmunity – Most authors thought that OLP is the autoimmune disorder in which mucosal epithelial cells are destroyed by T cells[1]. OLP is found in the patients with some autoimmune disorders such as primary biliary cirrhosis, chronic active hepatitis, ulcerative colitis, myasthenia gravis and thymoma[36].

(6) Systemic diseases – Some studies suggested the association of OLP with diabetes mellitus[37] and thyroid dysfunction[38].

In addition, there are other causal factors that have been mentioned to be associated with OLL[39].

(1) Dental materials – A number of dental restorative materials such as amalgam, gold, cobalt, palladium, nickel, chromium, composite and acrylic have been reported to be linked to the lesions similar to OLP both clinically and histologically which is called oral lichenoid contact lesion (OLCL)[40, 41].

(2) Medications – Drug reactions may trigger the lesions resembling OLP that is named oral lichenoid drug reaction (OLDR). These medications include anti-hypertensives (β-adrenergic blocking agents, angiotensin-converting-enzyme (ACE) inhibitors and diuretics), oral hypoglycemics, anti-hyperlipidemics (statins), non-steroidal anti-inflammatory drugs (NSAIDs), anti-malarials, anti-microbials, penicillamine, carbamazepine, hepatitis B vaccine, etc[40, 42].

(3) Graft-versus-host disease (GVHD) – Patients with chronic GVHD typically manifest the oral lesions similar to OLP both clinically and histologically which is called oral lichenoid lesion of GVHD (OLL-GVHD)[40, 43].

Immunopathogenesis

The pathogenesis of OLP was classically explained as an antigen-specific mechanism and a non-specific mechanism. Th1 cells and cytotoxic T cells (CTL) were the key components of the first mechanism, whilst mast cells were the key components

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of the second one. Sugerman et al. harmonized these 2 mechanisms and proposed a unifying hypothesis for the pathogenesis of OLP[8].

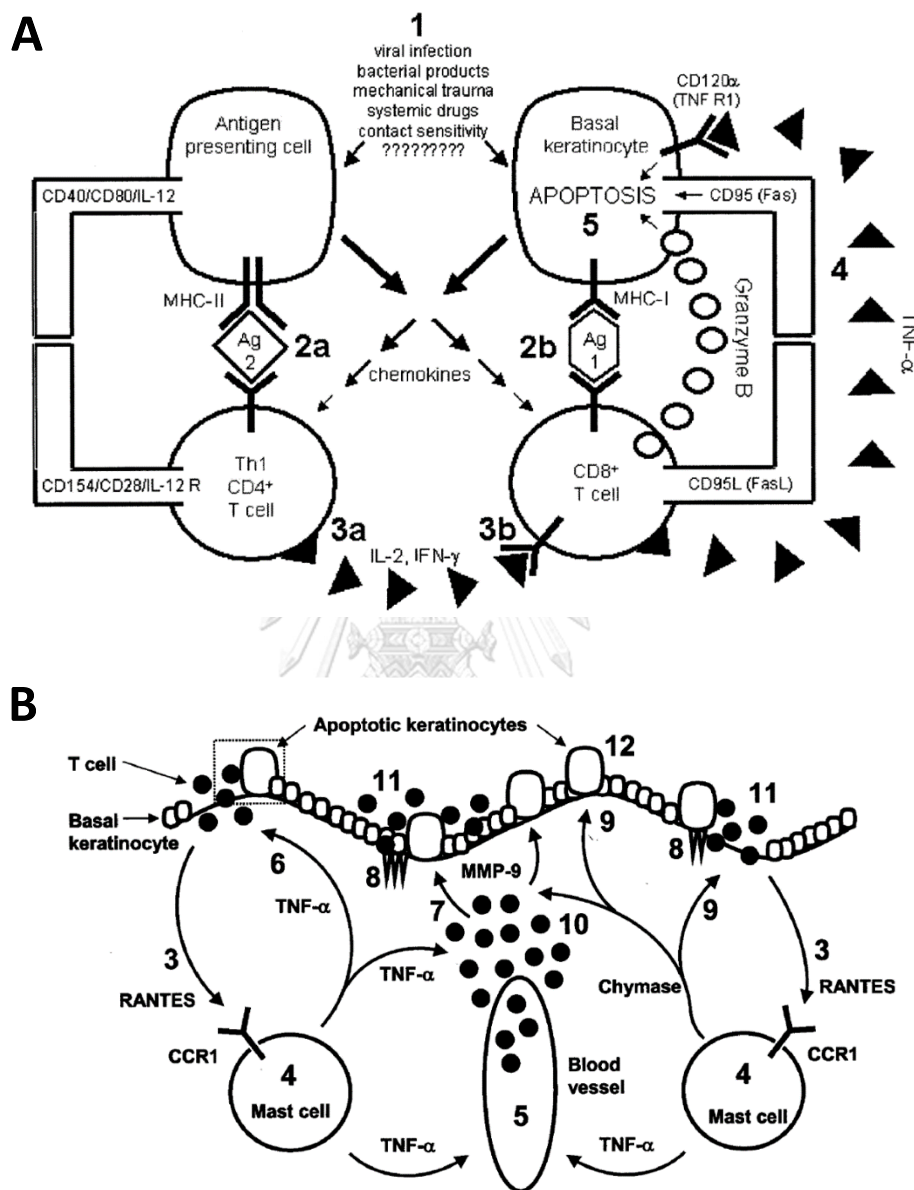

Figure 3 A unifying hypothesis for the pathogenesis of OLP[1, 8]

At the OLP lesional site, the basal epithelial cells express OLP antigens with major histocompatibility complex (MHC) class I molecules to CD8+ T cells, meanwhile, the epithelial cells and Langerhans cells possibly express the OLP antigens with MHC class II molecules to CD4+ T cells. The OLP antigens may be viral particles,

bacterial products, self-antigens, contact allergens or systemic medications. Th1 cells possibly help activation of $CD8^+$ T cells by secreting IFN-γ and interleukin-2 (IL-2) which subsequently bind to their receptors on $CD8^+$ T cells. Therefore, CTLs are activated and trigger the apoptosis of the basal epithelial cells via 3 possible mechanisms consisting of T cell-derived TNF-α binding TNFR1 on epithelial cell surfaces, T cell-derived granzyme B entering epithelial cells via perforin-induced membrane pores, and FasL on T cell surfaces binding Fas on epithelial cell surfaces. After the activation, CTLs undergo clonal expansion as well as release Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) (or chemokine (C-C motif) ligand 5 (CCL5)) and other cytokines. These cytokines upregulate mast cells to express C-C chemokine receptor type 1 (CCR1) and induce them for intra-lesional migration and degranulation releasing TNF-α. TNF-α from mast cells upregulates expression of endothelial cell adhesion molecules in blood vessels for T cell adhesion and extravasation. It also stimulates intra-lesional T cells to release RANTES and matrix metalloproteinase-9 (MMP-9) which is a proteolytic enzyme involving degradation of extracellular matrix. Furthermore, activated intra-lesional T cells and possibly the epithelial cells release a number of chemokines that attract extravasated T cells toward the OLP lesion. Eventually, the epithelial basement membrane is destroyed directly by chymase released from degranulation of mast cells and indirectly by stimulation of MMP-9 secretion from intra-lesional T cells. The breakdown of the epithelial basement membrane enables intra-lesional T cells to migrate into the epithelium and cause depletion of cell survival signals from the basement membrane to the epithelial cells. Hence, further epithelial cells undergoing apoptosis will proceed[1, 8] *[Figure 3]*.

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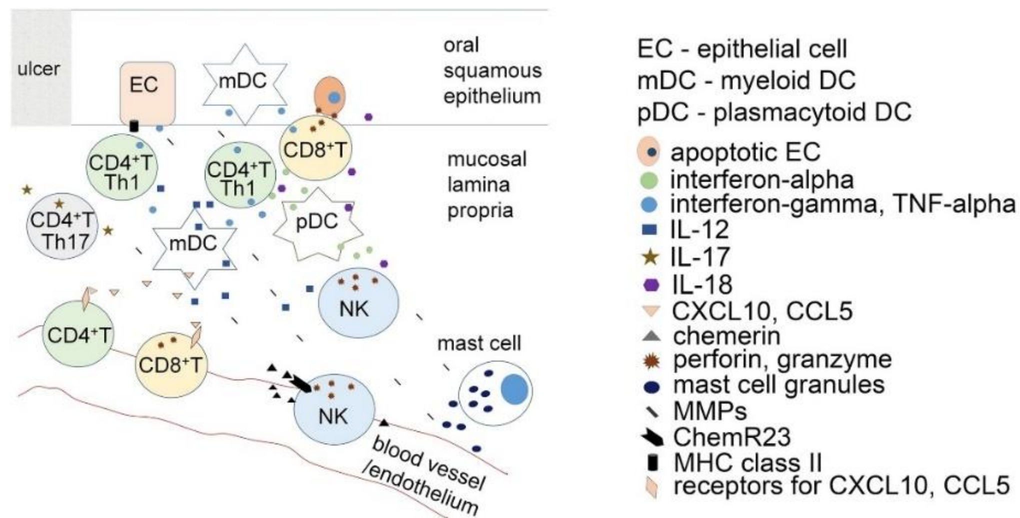

Figure 4 A hypothetical model of interactions implicated in OLP pathogenesis[6]

A hypothetical model of interactions implicated in OLP pathogenesis which recently introduced by Kurago still focuses on the mechanism described by Sugerman et al. However, the novel hypothesis also ascribes functions of natural killer (NK) cells and plasmacytoid dendritic cells (PDCs) in the pathogenesis. NK cells have abilities to mediate cytotoxicity and to produce IFN-γ, but their roles in OLP are uncertain. PDCs interact directly with lesional T cells and efficiently produce large amounts of type I IFN mainly IFN-α to activate other immune cells[6]. They also express granzyme B, an effector molecule involved in cytotoxicity[44] *[Figure 4]*.

T cells have long been considered the key mediators in the pathogenesis of OLP. T cells show predominant infiltrate in the mucosa of the patients with OLP than healthy individuals[8, 45, 46], but this different distribution is not observed in the peripheral blood[47, 48]. In the lesional mucosa, $CD8^+$ T cells are approximately twice as numerous as $CD4^+$ T cells[47, 49]. The majority of T cells infiltrating in the epithelium and in the lamina propria close to the disrupted basal cell layer are $CD8^+$ T cells. In contrast, in the deeper lamina propria, $CD4^+$ T cells are the main population[50]. Density of $CD8^+$ T cells in the lamina propria are increased in the zones of basement membrane disruption compared to the zones of basement membrane continuity; however, the density of $CD4^+$ T cells do not differ between those 2 zones[51].

Other T cells involved in the pathogenesis of OLP are Th17 cells and Tregs. Some studies showed the elevation of Th17 cells in the atrophic/erosive OLP compared

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to those in the reticular OLP. Level of IL-17, which is a cytokine secreted by Th17 cells, is increased in the erosive OLP lesion as well. IL-17 may play a role as an initiator or a consequence of mucosal erosion in OLP[6, 52, 53]. Regarding Tregs, a previous study found a significant increase of them, but a remarkable decrease of transforming growth factor-β (TGF-β) which is their cytokine in both reticular and erosive OLP. The authors postulated that Tregs in OLP increased in number, but impaired in their function for suppressing the inflammation[9]. Another study on Tregs in OLP could identify only a few positive cells in the OLP lesions[10]. On the other hand, plasma cells and antibodies have not been found to be implicated in the pathogenesis of OLP[8].

T cell-mediated immunity

Cell-mediated immunity comprises T cells as the key components. T cells are distinguished from other lymphocytes by their cell surface molecules that participate in antigen recognition, designated T cell receptors (TCRs). TCR is assembled together with CD3 molecule and ζ-chain to establish TCR complex, a pivotal structure in initiating T cell activation[54].

Development and activation of T cells

T cells originate from the hematopoietic stem cells in the bone marrow. Immature T cells then leave the bone marrow and migrate to a thymus via blood circulation for maturation. Once completed, mature naïve T cells leave the thymus and travel through the blood circulation to the secondary lymphoid organs[55]. In the secondary lymphoid organs, mature naïve T cells encounter numerous antigen presenting cells (APC) especially dendritic cells which carry antigens from the inflamed or injured peripheral tissues, such as skin or mucosa, and migrate through lymphatic vessels to the draining lymph nodes. As T cells interact with the specific antigens presented by dendritic cells, they are activated then undergo the clonal expansion and differentiation into effector T cells[56, 57]. However, if mature naïve T cells do not recognize any antigens, they will return to the bloodstream via the lymphatic drainage, circulate to the different peripheral sites of the body and come back to the lymph nodes again[58].

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Naïve $CD4^+$ T cells require 2 signals from the dendritic cells to drive them to become completely activated. The first signal is an antigen-specific signal which is delivered through the interaction of TCRs on T cell membranes and antigenic peptide/MHC class II molecules from dendritic cells. The second signals are co-stimulatory signals which are provided by the interaction between co-stimulatory molecules on the membranes of both T cells and dendritic cells. The co-stimulations between CD28 on T cells with CD80 (B7-1) or CD86 (B7-2) on dendritic cells and likewise between CD40L on T cells with CD40 on dendritic cells are important. The activation of naïve $CD8^+$ T cells similarly relies on these 2 signals, but they require MHC class I molecules instead of MHC class II molecules[59]. At some point, they require some cytokines from Th cells to collaborate on their proliferation and differentiation[60].

Differentiation and functions of T cells

Naïve $CD4^+$ T cells differentiate into Th cells with a variety of their subsets. Th cells play an essential role in secretion of numerous cytokines that help promote, regulate or suppress activities of other immune cells. Nowadays, Th cells are divided into 7 subsets consisting of Th1 cells, Th2 cells, Th9 cells, Th17 cells, Th22 cells, Tregs and T follicular helper (Tfh) cells[61]. Considering naïve $CD8^+$ T cells, they differentiate into CTLs with an important function involving the target cell destruction[62].

After the differentiation, effector T cells circulate through the bloodstream and extravasate from the blood vessels into the peripheral tissues by attraction of chemotactic factors from those sites[63]. Effector T cells recognize their specific antigens which are presented with MHC class I or MHC class II molecules by APCs, then they are activated again. As a result, they express their adhesion molecules to retain themselves at those sites and to perform their effector functions. Some effector T cells that recognize no specific antigen will die in the tissues or return to the circulation through the lymphatic vessels[55].

Th1 cells act against intracellular bacteria and protozoa. They secrete the cytokines; IFN-γ, IL-12 and TNF-α which can promote macrophage activation, nitric oxide production and CTL proliferation. By contrast, Th2 cells act against extracellular parasites. They produce diversified cytokines; IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13

which stimulate antibody production, eosinophil accumulation, but inhibit the functions of phagocytic cells[64, 65].

The effector function of CTLs is the direct killing of the infected or tumor cells via 3 main mechanisms. The first mechanism is the secretion of TNF-α and IFN-γ which are the anti-viral and the anti-tumor cytokines. The second mechanism is the production and secretion of cytotoxic granules containing perforin and granzyme. Perforin creates the pores in the membranes of the target cells and granzyme enters the cells to induce the apoptosis. The third mechanism is the expression of FasL to bind Fas molecules on the target cell surfaces. Fas/FasL interaction induces caspase cascades of the target cells, and likewise, it results in apoptosis[62].

Since the pathogens are cleared, more than 90% of antigen-specific effector T cells undergo apoptosis in a contraction phase. A few population of survival cells preserve their memory of the previously recognized pathogens and establish a long-term memory pool of memory T cells which are usually defined as $CD45RO^{+}$ cells[66].

Memory T cells

Memory T cells are generated throughout human life and accumulate with age[67]. They can survive for several months or years in a long-term quiescent phase without persistence of the antigens[68]. Maintenance of memory T cells for the long term depends on 2 main cytokines, namely IL-7 and IL-15 which promote their slow proliferation and help increase the level of anti-apoptotic proteins[69].

In contrast to their naïve counterpart, memory T cells respond more quickly to the previously encountered antigens, because they respond to a lower concentration of the antigens and a wider range of APCs, less depend on the co-stimulatory signals, exhibit higher functional avidity, require shorter duration of the stimulation and can proliferate more rapidly[70]. They express different adhesion molecules and chemokine receptors like CCR4 and CCR10 that enable them to migrate into the peripheral tissues supporting boarder distribution and antigen recognition at those sites[71]. A number of memory T cells specific for any antigens are larger than a number of naïve T cells specific for the same ones[55]. This specific property enables memory T cells to serve as a vigorous immunologic response to the re-encountered antigens[72].

At present, memory T cells are composed of 4 subtypes which vary in CD molecules, chemokine receptors and cytokine production. The first 3 subtypes include stem cell memory T (T_{SCM}) cells, central memory T (T_{CM}) cells and effector memory T (T_{EM}) cells, which are recognized as circulating memory T cells. The last one is T_{RM} cells which are the majority of memory T cells that occupy the peripheral tissue without recirculation[73, 74] *[Table 2]*.

Table 2 Human memory T cell subsets[73]

	T_{SCM} cell	T_{CM} cell	T_{EM} cell	T_{RM} cell
CD45RA	+	-	-	-
CCR7	+	+	-	-
CD69	-	-	-	+
CD103	-	-	-	+/-
IL-2	+++	+++	++	+/-
IFN-γ	+	++	+++	+++
TNF	+	++	+++	+++

+ low expression levels, ++ medium expression levels, +++ high expression levels, - no expression

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Circulating memory T cells recirculate in the bloodstream and extravasate into the peripheral tissues when challenged with antigens. Without their specific antigens, they exit the peripheral sites and enter the secondary lymphoid organs via the afferent lymphatic vessels and return to the blood circulation via a thoracic duct. In contrast, most T_{RM} cells reside within the particular peripheral tissues and do not recirculate[14]. Circulating memory T cells play an essential role in the control of systemic infections, but they often provide limited protection against the antigens localizing in the peripheral tissues[75]. Because circulating memory T cells, especially $CD8^+$ cells, poorly express homing molecules and chemokine receptors that are responsible for the extravasation and tissue infiltration in the absence of the persisting antigens[76]. Thus, the establishment of T_{RM} cells are required for the proper control of the pathogens[77].

Cross-reactivity of memory T cells

Remarkably, although a classical concept believed that memory T cells had a feature of antigenic specificity. To date, compelling evidences have indicated that they really present cross-reactivity to antigenic epitopes not previously encountered[73]. The cross-reactivity is a phenomenon that an individual TCR can recognize more than one peptide/MHC molecule[78]. Thus, a single T cell clone can recognize over a million of different peptides in the context of a single MHC molecule[79]. The peptide antigens from the viral particles often trigger the cross-reactivity. Some virus-specific memory T cells exhibit the cross-reactivity to alloantigens, autoantigens and unrelated pathogens[73].

The cross-reactivity of the T cells lead to both positive and negative effects. Cross-reactive T cells that recognize none of self-peptides provide an effective immune system by allowing a limited number of T cells to protect against almost all foreign antigens[80, 81]. The most deleterious effect from the cross-reaction is the autoimmune response. Weakly-autoreactive T cells that passed a selection process in the thymus may be activated by the peptides from the infectious antigens such as viruses that has cognate structures with the self-peptides and lead to the autoimmune diseases or acceleration of the previously initiated autoimmune responses[80, 82]. Cross-reactive memory T cells may exert the pathomechanism of LP by a mean that memory T cells specific for the formerly infected viruses may cross-react with other antigens, including contact allergens, medications, self-antigens as well as other heterologous viruses, and induce damage to the epithelial cells[2].

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Tissue resident memory T cells (T_{RM} cells)

T_{RM} cells were officially reported for the first time in 2001[83]. They are the non-recirculating population of memory T cells that resides in the previously infected or particular antigen encountered peripheral tissues in the long term to provide the locally rapid protective functions against the specific antigens. T_{RM} cells can persist in the particular peripheral tissues for several months or years[11, 12]. These sites include skin especially in the epithelial layer and hair follicle, intestine predominantly in the epithelial layer, lungs, female reproductive tract, salivary glands, lymph nodes and

brain. They are also found in the medulla of the thymus following the infections[14]. In the oral cavity, previous studies demonstrated localization of T_{RM} cells in both the epithelium and the lamina propria of the buccal mucosa[17] and the gingival tissue[84, 85]. This resident nature of T_{RM} cells is best represented in $CD8^+$ T cell subset rather than in $CD4^+$ T cell subset[14].

Generation of T_{RM} cells

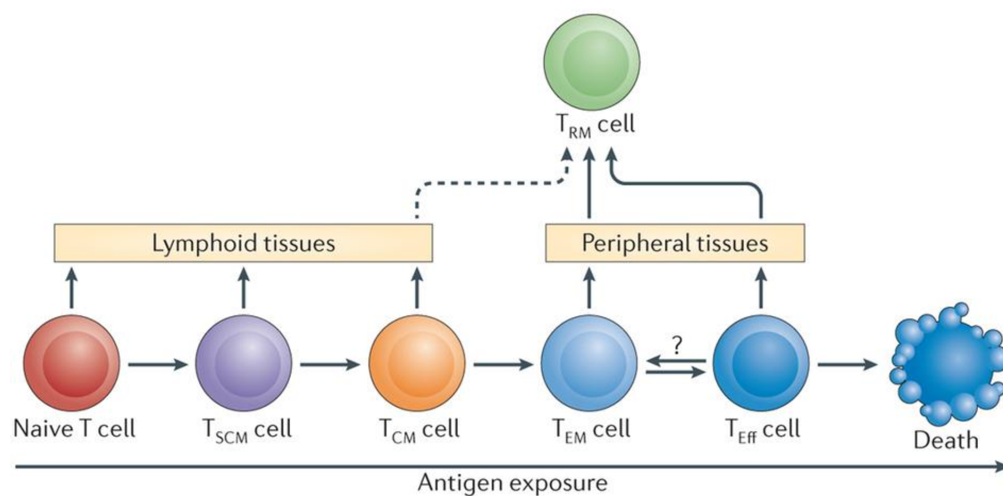

Figure 5 A model for the generation of T_{RM} cells[73]

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The key precursors for generating T_{RM} cells may be the effector T cells. After remission of the localized infection or the inflammation at the skin, effector T cells which have infiltrated into these sites may be terminated by 3 pathways. The majority of these cells undergo apoptosis in situ. Another group differentiates into circulating memory T cells and exits the tissue via the lymphatic vessels in a CCR7/CCL21-dependent pattern to return to the blood circulation. The minority of these cells with the signals from TGF-β enter the epithelium and develop into T_{RM} cells[72]. However, T_{EM} cells and T_{CM} cells may have capability to differentiate into T_{RM} cells as well[73]. In addition, a number of T_{RM} cells also proliferate in situ from pre-existing T_{RM} cells in response to local antigen encounter and do not migrate out of their residential areas[86]. There was a study claiming this autonomous proliferation substantially contributed to a boost of the secondary T_{RM} cell population[87]. The newly generated T_{RM} cells will not displace the pre-existing T_{RM} cells after subsequent infections; therefore, the T_{RM} cell

pool with multiple specificities can be stably maintained within the peripheral tissues for a long time[86].

Notably, T_{RM} cells can be generated and maintained in the skin and the mucosa without the local antigenic presentation. Only the local inflammation in the skin and the mucosa can cause enhanced attraction of effector T cells and differentiation into the T_{RM} population[75]. These cells are concentrated at the sites of prior infection or inflammation and they decrease in density at distant areas[88]. The maintenance of T_{RM} cells is generally determined by their longevity rather than by regular proliferation[72] *[Figure 5]*.

Functions of T_{RM} cells

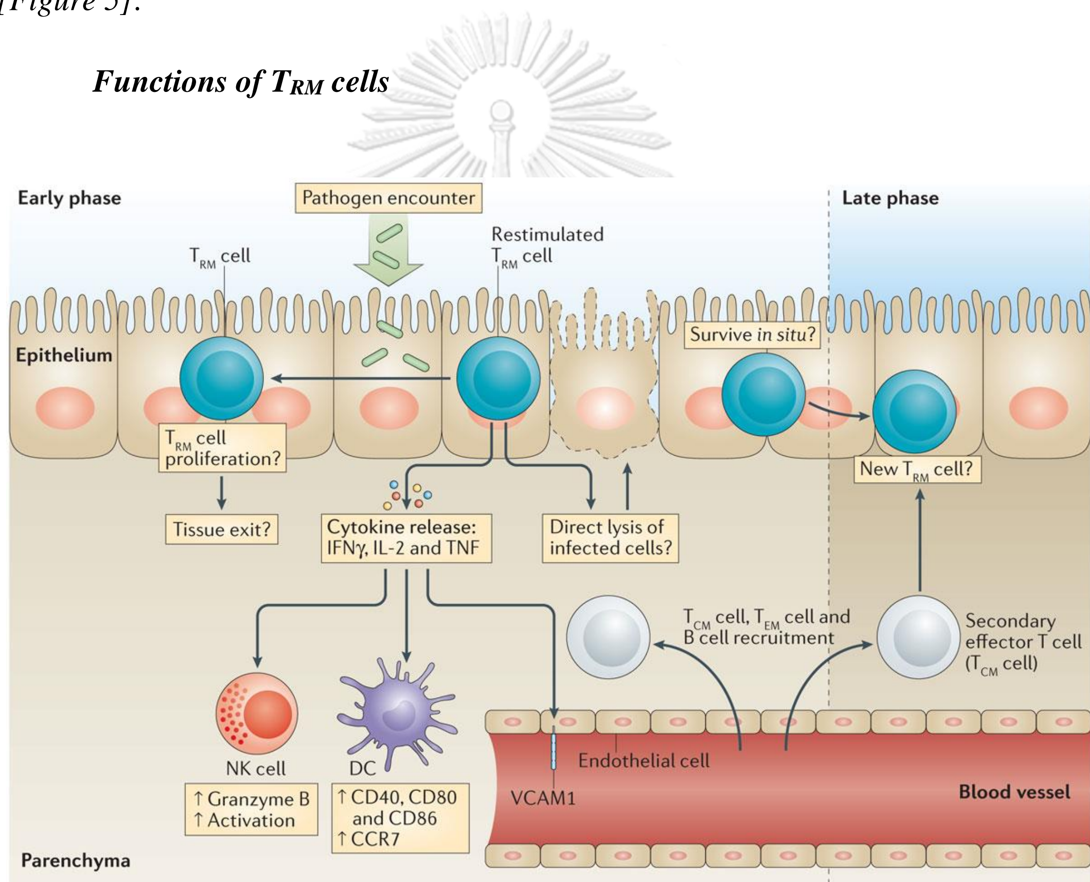

Figure 6 Protective functions of T_{RM} cells against local secondary infections[14]

The exact protective mechanisms of T_{RM} cells remains unclear. In the peripheral tissue like skin, an early phase of defense is launched when T_{RM} cells encounter the specific antigens and secrete some cytokines including IFN-γ, IL-2 and TNF-α. These cytokines attract dendritic cells and induce their expression of CD80, CD86 and CD40 which are the co-stimulators for T cell activation and CCR7, a lymph

node homing receptor. The cytokines also enhance NK cell recruitment and granzyme B releasing. They can induce the upregulation of vascular cell adhesion molecule 1 (VCAM1) in the local blood vessels for promotion of the extravasation of memory T cells and B cells into the peripheral tissues. In addition, T_{RM} cells can directly kill the infected cells via releasing perforin and granzyme. Thus, T_{RM} cells prompt rapid in situ defensive responses that help eliminate the pathogens[14]. Note that, at the same time, T_{RM} cells also undergo proliferation in situ after encountering the pathogens[86].

In a later phase several days later, circulating memory T cells are reactivated in the secondary lymphoid organs. They then proliferate and generate a great number of secondary effector T cells, chiefly T_{CM} cells that are recruited to the inflamed sites and differentiate into T_{RM} cells following the clearance of the pathogens[14] *[Figure 6]*.

T_{RM} cells show more potent effective functions than the circulating T cells[89]. T_{RM} cells in the skin exhibit slow dynamic migration through the epidermis with continuous transfiguration of their dendritic projection in shape, size and direction[90]. They usually contact with other cells localizing in the skin including keratinocytes, Langerhans cells and probably intraepithelial lymphocytes mainly γδ T cells to provide mandatory survival or stimulating signals[88]. This migratory pattern advocates their long-lived residence at the peripheral tissues and increase the efficacy of exploration and detection for the antigens[90]. Thereby, T_{RM} cells have been presumed as the first line of defense against the invasion of pathogens at the specific tissue surfaces before driving further adaptive immune responses[12, 88].

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Surface markers of T_{RM} cells

Discrimination of T_{RM} cells from other immune cells depends on 2 molecular markers expressed on their surfaces; CD69 and CD103. Both of them are essential for appropriate development and persistence of T_{RM} cells in the skin[13].

CD69 binds and blocks function of sphingosine-1-phosphate receptor 1 (S1PR1) that modulate the emigration of T_{RM} cells from the peripheral tissues and thus support stationary nature of T_{RM} cells[91]. Upregulation of CD69 expression depends on TNF-α and type 1 IFN[92]. CD69 is also expressed by activated B cells[93], activated T cells[94], NK cells[95] and neutrophils[96].

However, expression of CD103 or αEβ7 integrins are more specific for T_{RM} cells[14]. CD103 is expressed in more than 95% on $CD8^+$ T_{RM} cells and about 45-55% on $CD4^+$ T_{RM} cells in the intraepithelial layer of the intestinal mucosa[97]. They are also expressed in the skin, but they are expressed less than 6% on circulating blood-derived T cells[98]. CD103 promotes the long-term persistence of T_{RM} cells in the peripheral tissues by interaction with E-cadherins which are the adhesion molecules exclusively expressed by the epithelial cells. The binding of CD103 and E-cadherins helps the adherence of T_{RM} cells to the surrounding epithelial cells within the surfaces of the skin or the mucosa[99]. However, T_{RM} cells in the human skin are not strictly required to express CD103. $CD103^+$ T_{RM} cells exhibit more potent effective functions, but less proliferative capability than $CD103^-$ T_{RM} cells[89]. The expression of CD103 is upregulated by the cytokine secreted by the epithelial cells called TGF-β which is necessary for the development and the maintenance of T_{RM} cells. TNF and IL-33 play a role in this induction as well[100].

Roles of T_{RM} cells in human diseases

With regard to human diseases, several studies showed persistent distribution of virus-specific T_{RM} cells in various peripheral tissues. Human lungs are resided with influenza-specific T_{RM} cells that protect the lungs from recurring influenza virus infection. T_{RM} cells provide rapid responses to these viruses via quick upregulation of perforin and granzyme B when they contact their specific antigens or expose to type I IFN, thus they play essential roles in the control of viral replication and dissemination[101]. Although T_{RM} cells exert paramount roles in the protective functions, they can elicit tremendous destructive outcomes mediated by cellular immune disorder[14].

Psoriasis is a prototype of a T_{RM} cell-mediated autoimmune cutaneous disease that lead to keratinocyte hyper-proliferation. It is characterized by classical manifestations of well-defined erythematous plaques with silvery-white scales. The lesions are completely resolved with immunosuppressive therapy, but often recur in the same places and growing again to their previous sizes once the therapy is discontinued[102]. Autoantigens are regarded to be the putative antigens[92]. The patients with psoriasis show accumulation of T_{RM} cells in the local inflamed skin lesions. Even

though the clinical lesions are healed for several years, these cells still persist in the resolved lesions with the ability to produce the inflammatory cytokines, IL-17 and IL-22, that play critical roles in the maintenance and potential elicitation of the recurrent disease. Furthermore, IL-17-secreted $CD8^+$ T cells and IL-22-secreted $CD4^+$ T cells, which promote the role of T_{RM} cells in the pathogenesis of psoriasis, are also retained in the resolved lesions[15].

Another best-characterized disease model that mediated by T_{RM} cells is fixed drug eruption. It is an allergic reaction that usually appears as well-defined erythematous macules or plaques on skin or mucosa. The lesions typically recur at exactly the same places after each ingestion of causative drugs even several years later after the last exposure to those drugs. Once the causative drugs are discontinued, the lesions spontaneously resolve, leaving the gray-brown hyperpigmented macules or plaques on the previous lesions[103]. Fixed drug eruption is characterized by $CD8^+$ intraepidermal T cells, the resident population of memory T cells, which reside in a primed stage in the resolved lesions over a prolonged period of time. Upon challenging by the particular drugs, they are induced to rapidly release a large amount of IFN-γ and cytotoxic granules into the environment leading to subsequent tissue destruction. IL-15 expressed from the lesional epidermis is essential for the long-term maintenance of $CD8^+$ intraepidermal T cells in the lesion of fixed drug eruption[11, 16].

T_{RM} cells in OLP

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The requisite factors for the localization of T_{RM} cells may comprise CD69 molecules, CD103 molecules, E-cadherins and TGF-β. A few studies have addressed the issue about CD69 in OLP, and they all considered CD69 to be an activation marker of T cell not a localization marker of T_{RM} cells. $CD8^+$ T cells in the OLP lesions expressed CD69 much more frequently than those in peripheral blood[104], but no significant difference in $CD69^+$ $CD8^+$ T cell expression was found between the lesions and normal mucosa[105]. In the OLP lesions, $CD69^+$ T cells predominantly resided in close proximity to the epithelium[106].

There has been only one study on CD103 in OLP in 1997. The study regarded CD103 as an important element for the localization of T_{RM} cells in the OLP lesions. For both the skin LP and OLP, the proportions of $CD103^+$ cells to $CD3^+$ cells in the

epithelium or the epidermis was higher than those in the lamina propria or the dermis. The increase in the proportions of $CD103^+$ cells in OLP was observed when compared to the normal mucosa, but this difference was not found between the skin LP and the normal skin[17].

Several studies have focused on E-cadherin in OLP, but they discussed in detail a malignant transformation marker rather than an adhesion molecule to T_{RM} cell. The decreased expression of E-cadherins was found in the OLP lesions compared to the normal controls[107, 108]. In the OLP lesions, E-cadherin expression was focally lost in the basal cell layer of the epithelium particularly in the zone of dense sub-epithelial lymphocyte infiltrate. By contrast, in non-diseased area of OLP and normal oral mucosa, E-cadherin showed pronounced expression in the basal and parabasal layer and reduced intensity in the superficial cell layer[107, 109]. The expression of E-cadherin in OLP was not correlated to histological characteristics or locations of the lesions[110]. However, one compelling study reported that OLP lesions showed 10-fold increase in E-cadherin expression over normal tissues[111].

Regarding TGF-β, some studies revealed that specimens of the OLP lesion and serum of the OLP patient showed lower levels of TGF-β than healthy controls[112, 113]. In the OLP lesion, T cells in the lamina propria showed variable expression of TGF-β, whilst those in the epithelium were totally negative for TGF-β expression[50]. On the other hand, one study stated that the expression of TGF-β in atrophic OLP was significantly higher than that in non-atrophic OLP and normal oral mucosa[114].

Up to now, the definite mechanism of T_{RM} cells in the pathogenesis of OLP has not been illustrated yet.

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CHAPTER III

MATERIALS AND METHODS

Sample size

$$n = \frac{(z_{1-\frac{\alpha}{2}} + z_{1-\beta})^2 (\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2} = \frac{(1.96+1.28)^2 (38.3^2 + 15.5^2)}{(60.5-25.7)^2}$$

$$= 14.80$$

A sample size needed for comparison of 2 independent means was determined using the above formula[115]. Means (μ) and standard deviations (σ) were obtained from the values of the previous study by Walton et al, 1997[17]. Significance level (α) and power (1-β) were set as 0.05 and 0.9 respectively. The calculated output was 14.80. Therefore, a total of 15 specimens from OLP lesions and 15 specimens from normal mucosa were decided to be utilized in this study.

Inclusion criteria

The diagnostic criteria for OLP used in this study were modified from the criteria defined by WHO in 1978[24] and van der Meij and van der Waal in 2003[27].

(1) Clinical manifestations showed bilateral white striae with atrophic and/or ulcerative/erosive lesions.

(2) Histopathological features presented a well-defined band-like zone of cellular infiltration confined to a superficial part of lamina propria, consisting mainly of lymphocytes and presented signs of liquefactive degeneration in a basal layer of epithelium with absence of epithelial dysplasia.

Exclusion criteria

(1) Lesions associated with an amalgam filling and/or a metal crown restoration.

(2) Patients had known history of systemic diseases and/or medication taking.

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(3) Patients received topical corticosteroid therapy for the lesions in the last 1 month prior to performing the biopsies.

(4) Patients were pregnant women, alcohol drinkers and/or smokers.

OLP tissue samples

This study utilized 15 specimens of OLP retrieved from paraffin-embedded tissue blocks from the Department of Oral Pathology, Faculty of Dentistry, Chulalongkorn University between 2011-2017. All of the specimens were taken from the buccal mucosa for the diagnostic purposes and were diagnosed both clinically and histopathologically as OLP by one clinician and one pathologist respectively. All biopsies were performed before initiating the corticosteroid therapy. Characteristics data of the patients were recorded including age, sex, medical history, chief complaint, duration of disease, involved areas, biopsy site and OLP type.

A total of 15 OLP patients constituted of 87% females and 13% males. The average age was 54.3 ± 11.46 years. The most common chief complaint is burning sensation (47%), followed by pain (33%) and feeling roughness (7%). A few patients experienced no symptom, but the lesions were incidentally detected by dentists (13%) *[Figure 7]*. Of the 15 OLP patients, 13 patients had mean duration of 6.5 ± 4.57 months, whereas the other 2 patients did not know when the lesions had erupted. The majority of OLP types around the biopsy site was ulcerative/ erosive (47%), followed by atrophic (40%) and reticular (13%) *[Figure 8]*. All of the OLP patients had the lesions at buccal mucosa (100%). The lesions also erupted at gingiva (73%), mucobuccal fold (53%), tongue (53%), hard palate (13%), lip (13%) and floor of mouth (7%) *[Figure 9]*. The OLP sections had an average area of 1.4 ± 0.55 mm^2. The average width of the lymphocytic bands was 385 ± 152.8 µm.

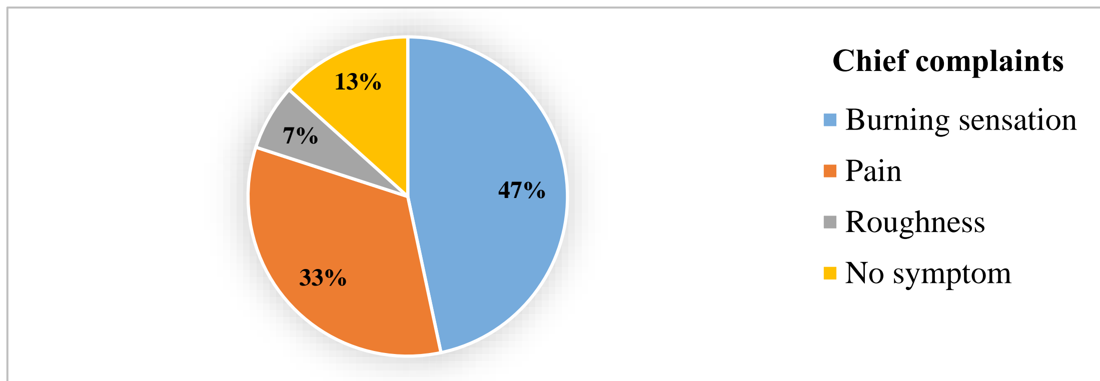

Figure 7 Distribution of chief complaints among OLP patients

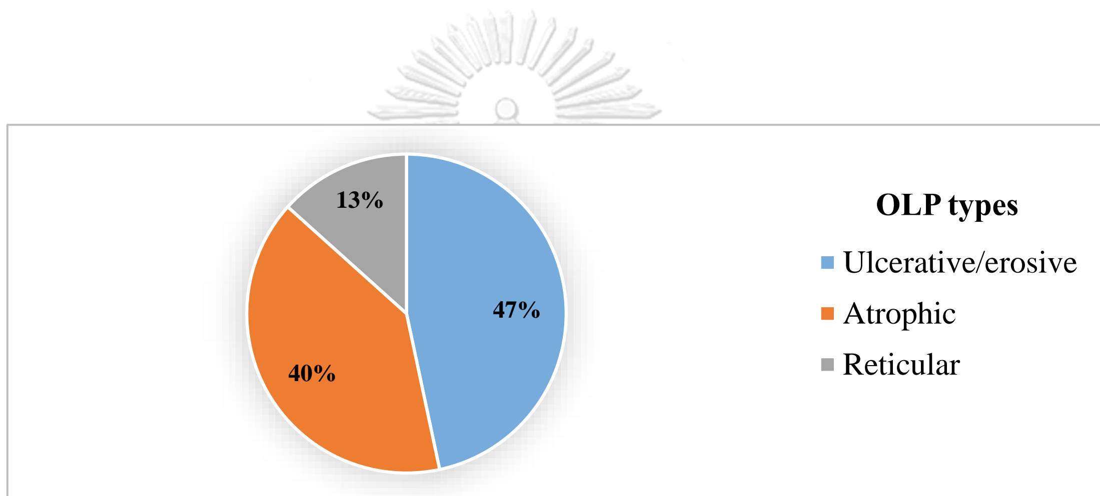

Figure 8 Distribution of types of OLP lesions among OLP patients

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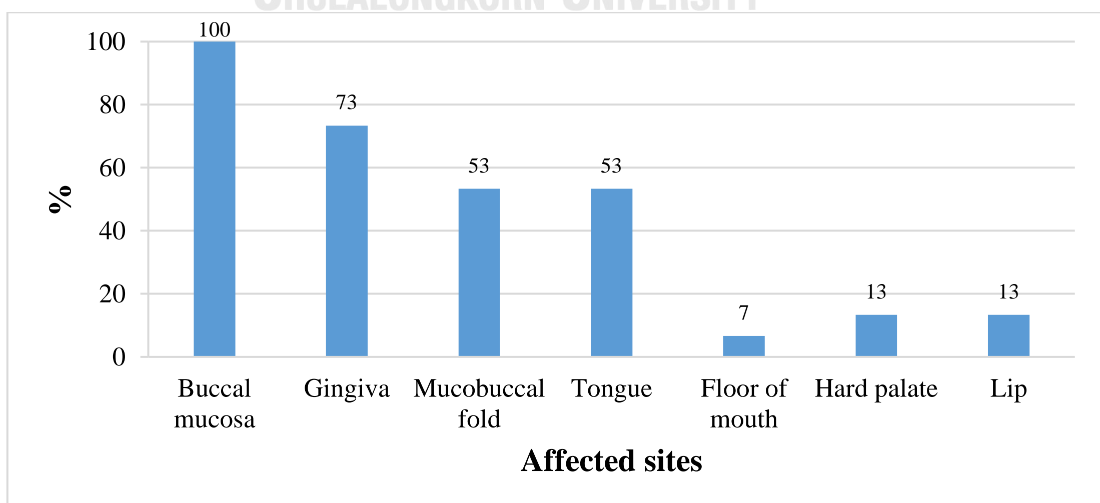

Figure 9 Distribution of sites of OLP lesions among OLP patients

Normal mucosa tissue samples

The normal mucosa was taken from 15 healthy normal subjects who were undergoing third molar surgical extractions at the Oral and Maxillofacial Surgery Clinic, Faculty of Dentistry, Chulalongkorn University. The biopsy specimens were obtained from their non-inflammatory mucosal area in the buccal region adjacent to the sites of the surgeries during the removal of impacted teeth. The specimens were fixed with 10% formalin for a maximum of 24 hours before embedded in the paraffin blocks at the Department of Oral Pathology. All of the healthy normal subjects had no known systemic diseases and currently did not take any medications. Informed consents were obtained from all of them prior to participating in this study.

A total of 15 healthy normal subjects comprised 87% females and 13% males. The mean age was 22.7 ± 5.16 years. An average area of the normal mucosa sections was 2.3 ± 0.90 mm^2.

Immunohistochemical study

Materials

- Primary anti-human antibodies included:
  - Anti-CD3 antibody - Rabbit polyclonal [A0452] (Dako, Glostrup, Denmark)
  - Anti-CD4 antibody - Rabbit monoclonal (EPR6855) [ab133616] (Abcam, Cambridge, UK)
  - Anti-CD8 antibody - Mouse monoclonal (4B11) [NCL-L-CD8-4B11] (Novocastra, Newcastle Upon Tyne, UK)
  - Anti-CD103 antibody - Rabbit monoclonal (EPR4166(2)) [ab129202] (Abcam, Cambridge, UK)
- Isotype-matched antibodies included:
  - N-universal negative control – mouse [N1698] (Dako, California, USA)
  - N-universal negative control – rabbit [N1699] (Dako, California, USA)

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- 3,3'-diaminobenzidine (DAB) chromogen kit: EnVision+ Dual Link System-HRP (DAB+) [K4065] (Dako, California, USA); the kit composed of:
  - Dual endogenous enzyme block
  - Labelled polymer-horseradish peroxidase (HRP)
  - DAB+ substrate buffer
  - DAB+ chromogen
- Other chemical supplies
  - Xylene
  - 100%, 95% and 80% Ethanol
  - Deionized (DI) water
  - Tap water
  - 0.5% Hydrogen peroxide in methanol
  - 1mM Ethylene diamine tetraacetic acid (EDTA) pH 8.0
  - Tris-buffered saline with Tween (TBST)
  - 5% Skim milk in Dulbecco's phosphate-buffered saline (DPBS)
  - Hematoxylin (Vector, California, USA)
  - Aqueous mounting medium (Dako, Glostrup, Denmark)

Methods

In this study, positive controls were human tonsils. Isotype controls were confirmed by replacing primary antibodies with non-binding isotype-matched antibodies.

The deparaffinization and rehydration were conducted as follows:

(1) Cut the paraffin-embedded specimens into serial slices with 2 µm in thickness and mounted the sections on glass slides.

(2) Placed the slides over a dry oven at 70°C for 1 hour and then allowed them to cool down for 30 minutes.

(3) Washed the slides with 3 changes of xylene for 5 minutes each

(4) Washed the slides with 2 changes of 100% ethanol for 2 minutes each

(5) Washed the slides with 2 changes of 95% ethanol for 2 minutes each

(4) Washed the slides with 80% ethanol for 2 minutes

(5) Washed the slides with tap water for 2 minutes

(6) Washed the slides with DI water for 2 minutes

The staining procedure was conducted as follows:

(1) Incubated the sections with 100 µl of 0.5% hydrogen peroxide in methanol for 10 minutes for blocking endogenous peroxidase activity.

(2) Washed the slides with tap water for 3 minutes.

(3) Immersed the slides in a staining dish containing 1mM EDTA pH 8.0, covered the dish with a lid and placed it in a pressure cooker for antigen retrieval at $90^{O}C$ for 30 minutes.

(4) When the pressure cooker was turned off, took the staining dish out and put it in tap water for 45 minutes to cooldown.

(5) Immersed the slides in DI water for 3 minutes.

(6) Drew a border around each section with a liquid blocker pen.

(7) Washed the slides with TBST for 5 minutes.

(8) Incubated the sections with 100 µl Dual endogenous enzyme block for 30 minutes in humidified chamber at room temperature for blocking endogenous peroxidase and alkaline phosphatase activity.

(9) Washed the slides with TBST for 3 minutes.

(10) Incubated the sections with 100 µl of 5% skim milk in DPBS for 30 minutes in humidified chamber at room temperature for blocking non-specific antibody binding.

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(11) Incubated the sections with 100 µl primary antibody in a single labelling step. Primary anti-human antibodies used in this study included anti-CD3 antibody (1:50), anti-CD4 antibody (1:150), anti-CD8 antibody (1:10) and anti-CD103 antibody (1:100).

(12) Placed the slides in a humidified chamber at $4^{O}C$ overnight.

(13) Washed the slides with 2 changes of TBST for 5 minutes each.

(14) Incubated the sections with 100 µl Labelled polymer-HRP for 30 minutes in the humidified chamber at room temperature.

(15) Washed the slides with 3 changes of TBST for 3 minutes each.

(16) Prepared DAB+ working solution by mixing 1 ml of DAB+ substrate buffer with 20 µl of DAB+ chromogen

(17) Incubated the sections with 100 µl DAB+ working solution for 1 minute approximately and checked stain intensity under a light microscope.

(18) Washed the slides with DI water for 5 minutes.

(19) Counterstained the sections with hematoxylin for 5 minutes.

(20) Washed the slides with DI water for 5 minutes

(21) Rinsed the slides with running tap water using at a low flow rate for 45 minutes and checked the stain intensity under the light microscope.

(22) Dried the slides in the air, then covered the section with 1 drop of aqueous mounting medium and mounted with cover slips.

(23) Sealed the cover slip edges with nail polish and allowed them to dry for 1 hour.

Quantitative analysis

The stained sections were scanned under the light microscope at 400x magnification with OLYMPUS dotSlide (Olympus Corporation, Tokyo, Japan). The scanned images were viewed via OLYMPUS OlyVIA, Version 2.9 (Olympus Corporation, Tokyo, Japan) and analyzed with ImageJ, Version 1.51j8 (National Institutes of Health, Maryland, USA).

The positive cells were defined as the cells that showed brownish stains of their cellular membranes with the staining intensity greater than or equal to the positive-stained cells in the positive tissue controls.

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The epithelium and the lamina propria were separately evaluated by one observer. The total number of the positive cells were counted through the entire area of the epithelium. In the lamina propria of the OLP samples, only the positive cells confined to the areas of the lymphocytic bands were counted. The examined areas in the lamina propria of the normal mucosa samples were determined by the length of their epithelium and the average width of the OLP's lymphocytic bands. The positive cells in these limited areas beneath the overlying epithelium were counted. The results were presented as the numbers of the positive cells per area (cells/mm^2) as well as calculated to the proportions of $CD4^+$ cells, $CD8^+$ cells and $CD103^+$ cells to the total number of $CD3^+$ cells (%).

Statistical analysis

Data were analyzed using IBM SPSS Statistics for Windows, Version 22.0 (IBM corp., New York, USA). Distribution of the characteristics data was described in frequencies. The numbers of the positive cells per area as well as the proportions of the positive cells to the total number of $CD3^{+}$ T cells were presented in mean ± SD.

An unpaired t-test was used to determine the difference in the values between the OLP group and the normal mucosa group. A paired t-test was used to compare the values between the epithelium and the lamina propria of each group. Alternately, a Mann-Whitney U test and a Wilcoxon Signed Ranks test were used if an assumption of normality evaluated by Shapiro-Wilk test was violated. A p-value less than 0.05 was considered statistically significant.

Ethical consideration

A study protocol was submitted for approval by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University [HREC-DCU 2017-093]. A request for permission to use the OLP specimens in this study was submitted to the Department of Oral Pathology, Faculty of Dentistry, Chulalongkorn University. All of the healthy normal subjects were given information about the research and also the nature, consequences and potential risks associated with the biopsy procedures. The informed consents were obtained from all of them prior to participating in the study.

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CHAPTER IV

RESULTS

Distribution of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells

The expression of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells were detected by means of single-labelling immunohistochemistry. In OLP sections, $CD3^+$ cells distributed dispersedly throughout the epithelium but slightly increased in the parabasal and the basal cell layers. Almost all $CD3^+$ cells located densely within the lymphocytic bands at the superficial lamina propria. Most sections demonstrated that the bands of $CD3^+$ cells in the lamina propria did not stay attached to the layers of the basal epithelial cells, but there were narrow spaces passing through these interfaces. Almost all of the positive cells were intensely stained around cell borders. Shapes and sizes of them were mostly homogeneous throughout the sections *[Figure 10]*.

$CD4^+$ and $CD8^+$ cells in OLP sections showed the distribution and the expression pattern in the same fashion as $CD3^+$ cells. Most of the sections expressed $CD8^+$ cells more than $CD4^+$ cells. Locations of $CD4^+$ cells were intermixed with locations of $CD8^+$ cells without specific grouping of each cell type. However, in the epithelium, $CD8^+$ cells were slightly more pronounced at zone of the basal cells *[Figure 11, 12]*.

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The quantities of $CD103^+$ cells from each OLP section were quite variable. In the epithelium, $CD103^+$ cells were mainly found in the lower half areas with dispersed distribution pattern. Most of them were also detected within or adjacent to the basal cell layers. In the lamina propria, the majority of $CD103^+$ cells dispersed within the areas of the lymphocytic bands. They also often appeared in epithelium-lamina propria interface zones *[Figure 13]*. Some $CD103^+$ cells presented at the locations that matched the locations of $CD8^+$ cells, while some shared the locations with $CD4^+$ cells. Most of the $CD103^+$ cells exhibited strong staining around cell borders, while some showed weaker staining. Shapes and sizes of them were rather similar *[Figure 11-13]*.

In the normal mucosa sections, $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells distributed sparsely throughout the epithelium and the superficial lamina propria. They were

infrequently found in the deep part of the lamina propria. Some sections demonstrated very scant CD103$^+$ cells. The positive cells, especially CD103$^+$ cells, were quite varied in shapes, sizes and staining patterns *[Figure 14-17]*.

The expression of CD3$^+$, CD4$^+$, CD8$^+$ and CD103$^+$ cells in the human tonsils as the positive controls were shown in *Figure 18-21* respectively. The images of the isotype controls in the OLP tissue, the normal mucosa tissue and the human tonsil tissue were shown in *Figure 22-24* respectively.

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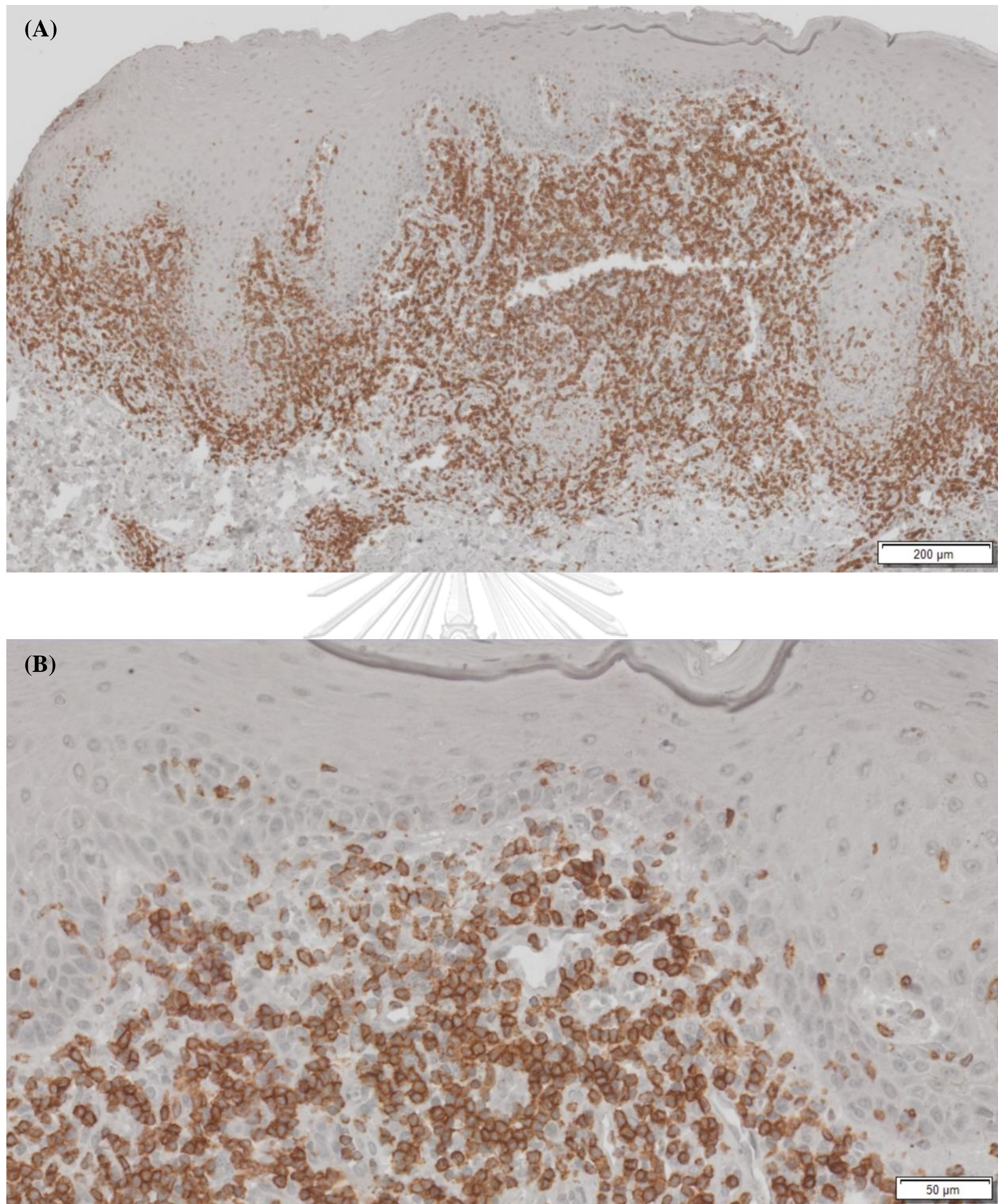

Figure 10 Immunohistochemical staining of $CD3^{+}$ cells in OLP tissue

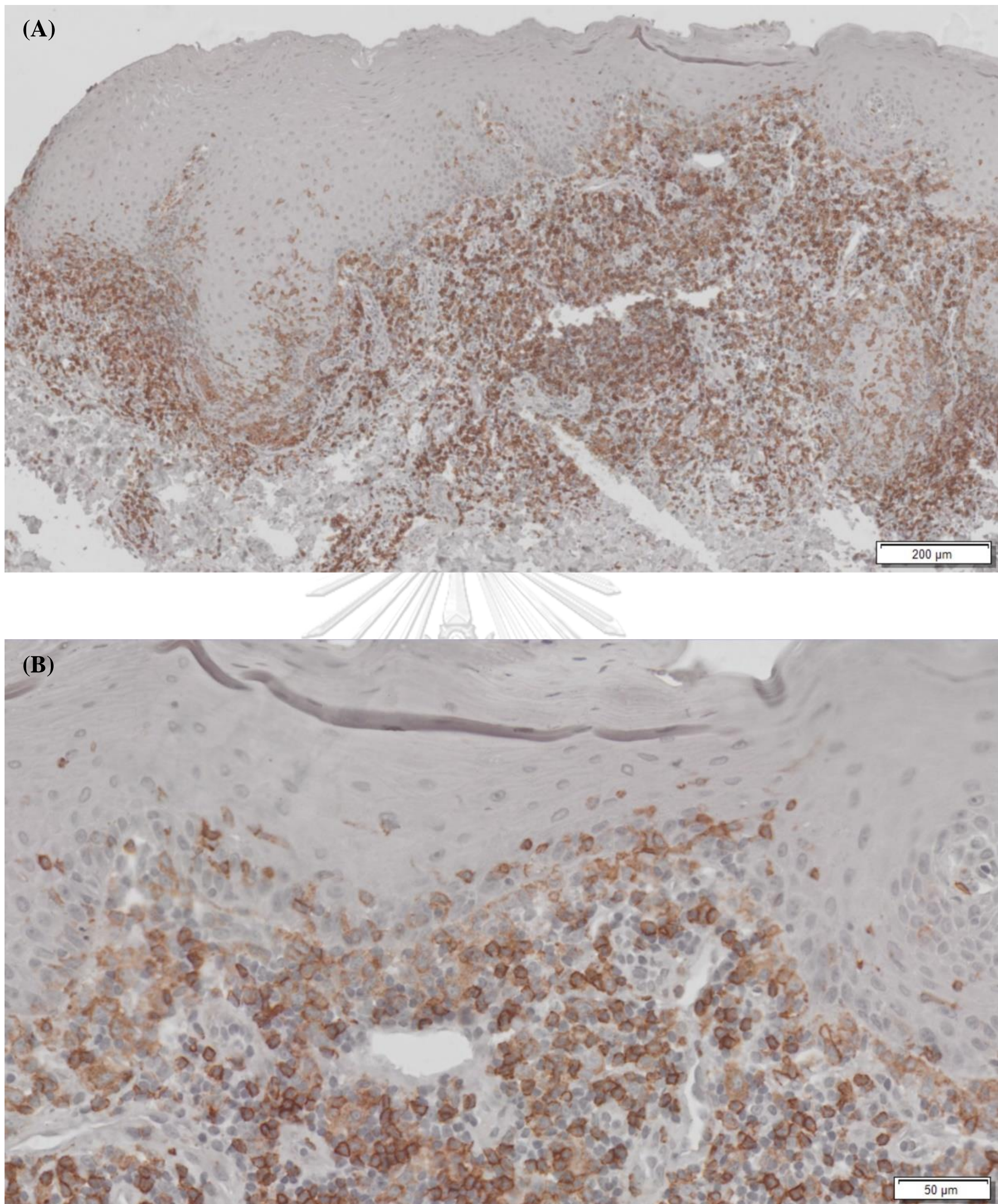

Figure 11 Immunohistochemical staining of $CD4^+$ cells in OLP tissue

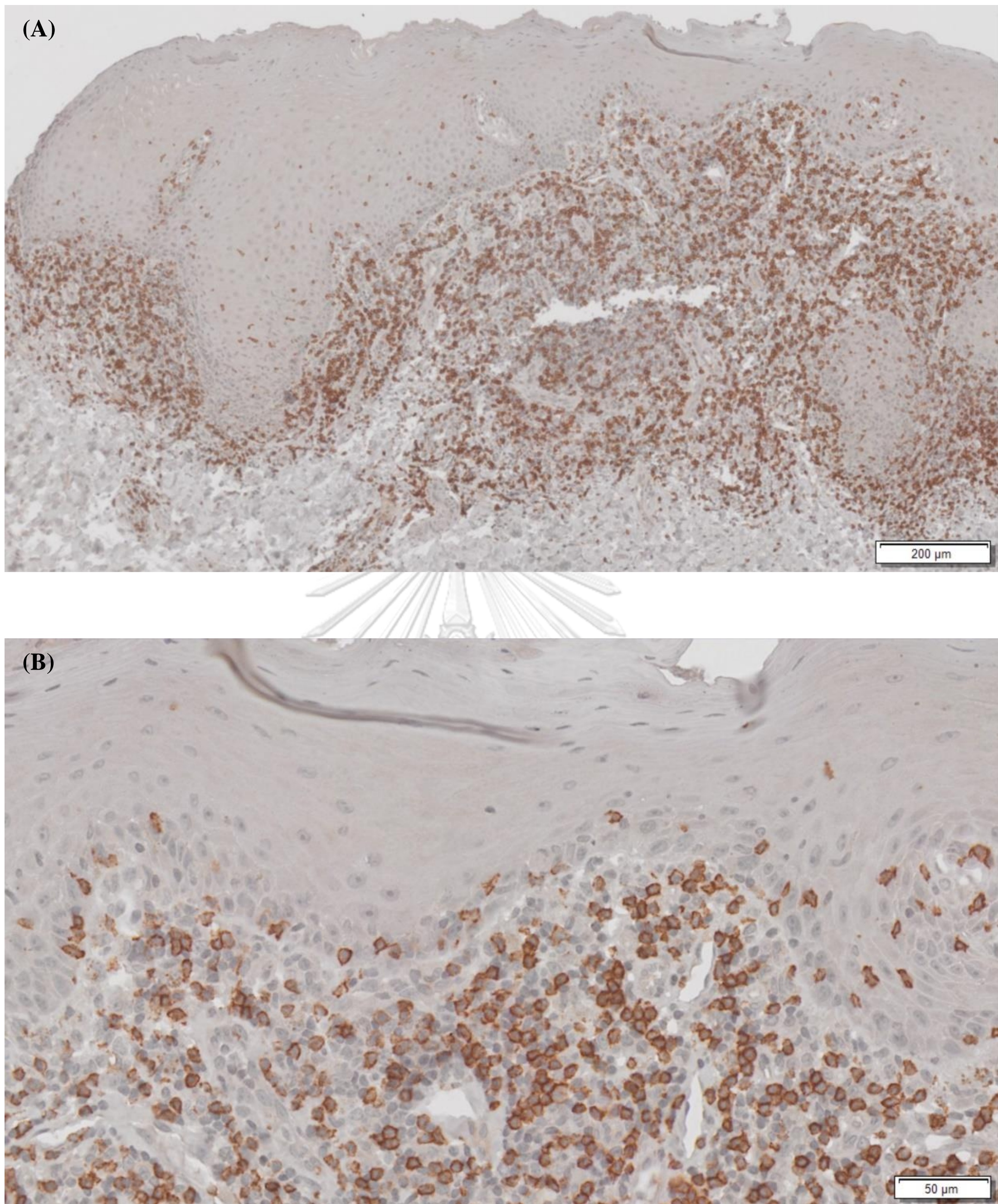

Figure 12 Immunohistochemical staining of $CD8^{+}$ cells in OLP tissue

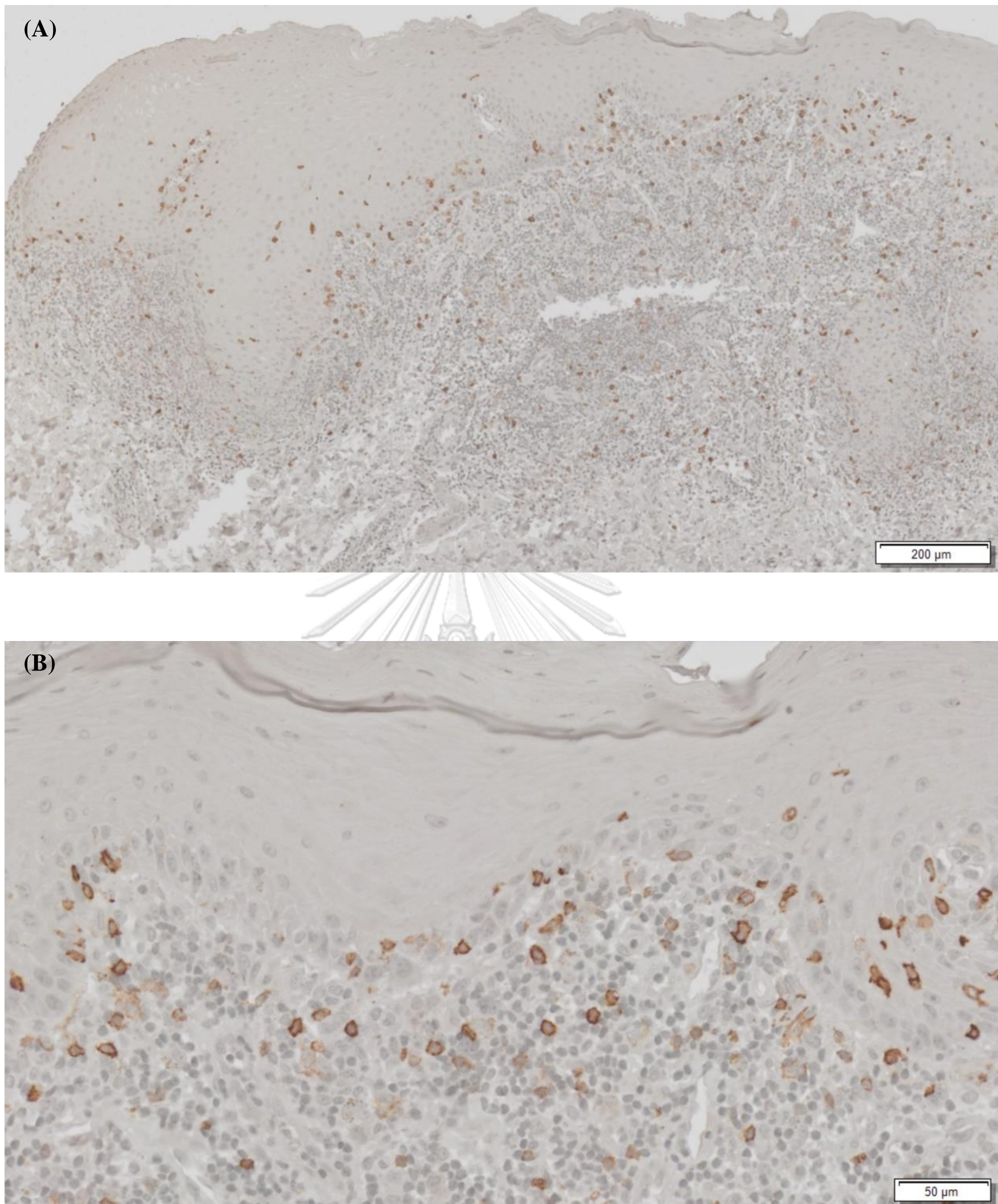

Figure 13 Immunohistochemical staining of CD103^{+} cells in OLP tissue

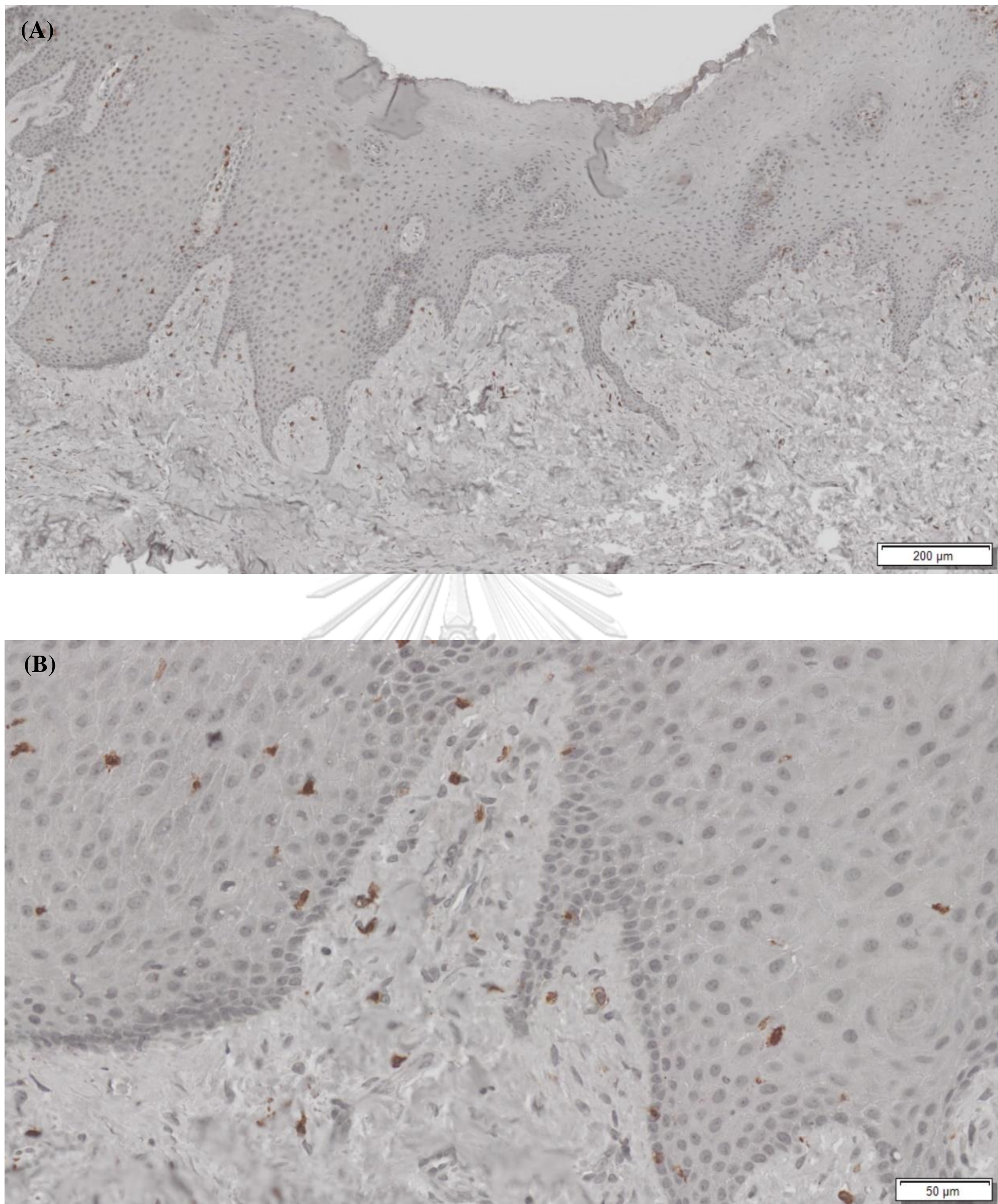

Figure 14 Immunohistochemical staining of $CD3^+$ cells in normal mucosa tissue

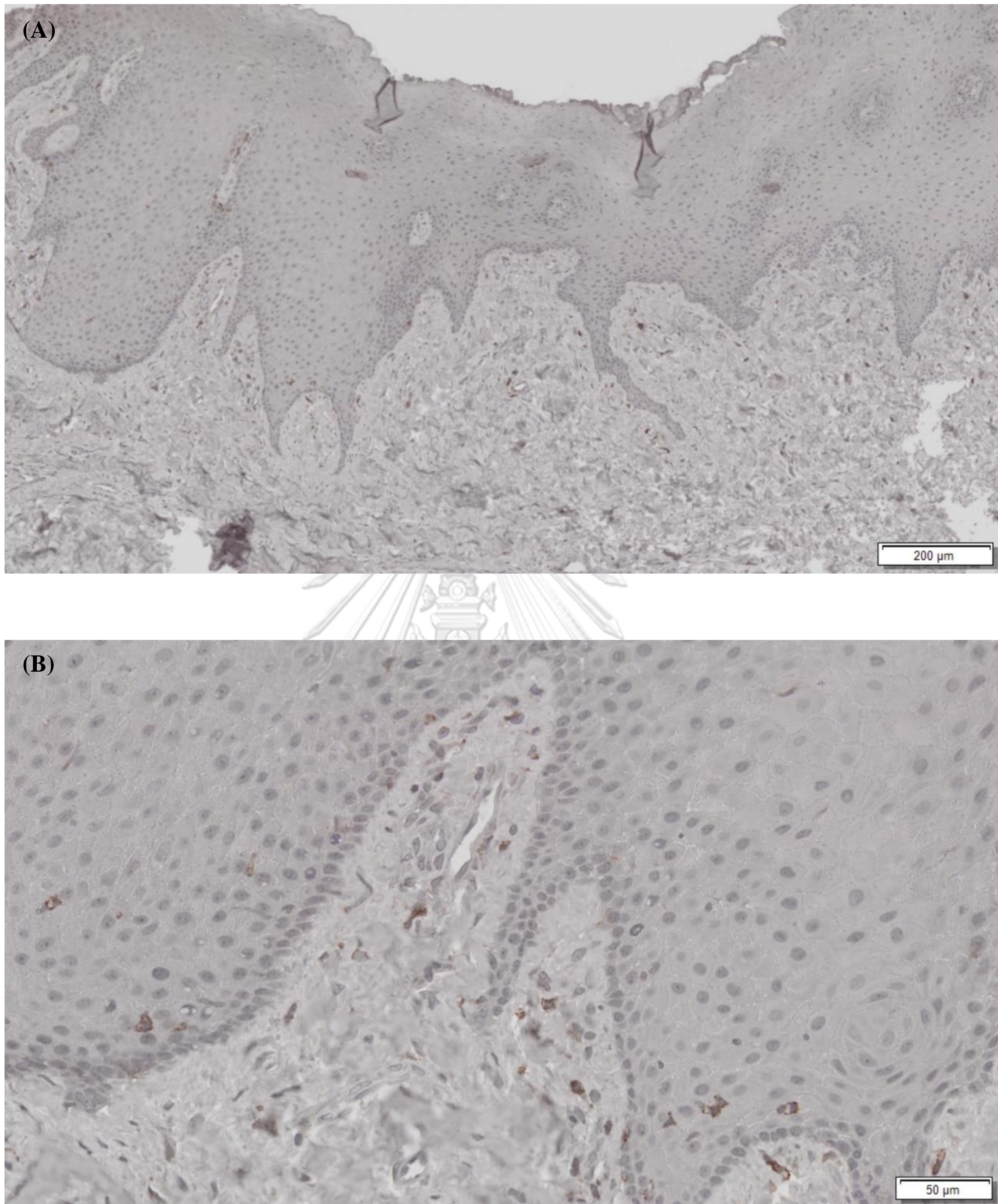

Figure 15 Immunohistochemical staining of CD4^{+} cells in normal mucosa tissue

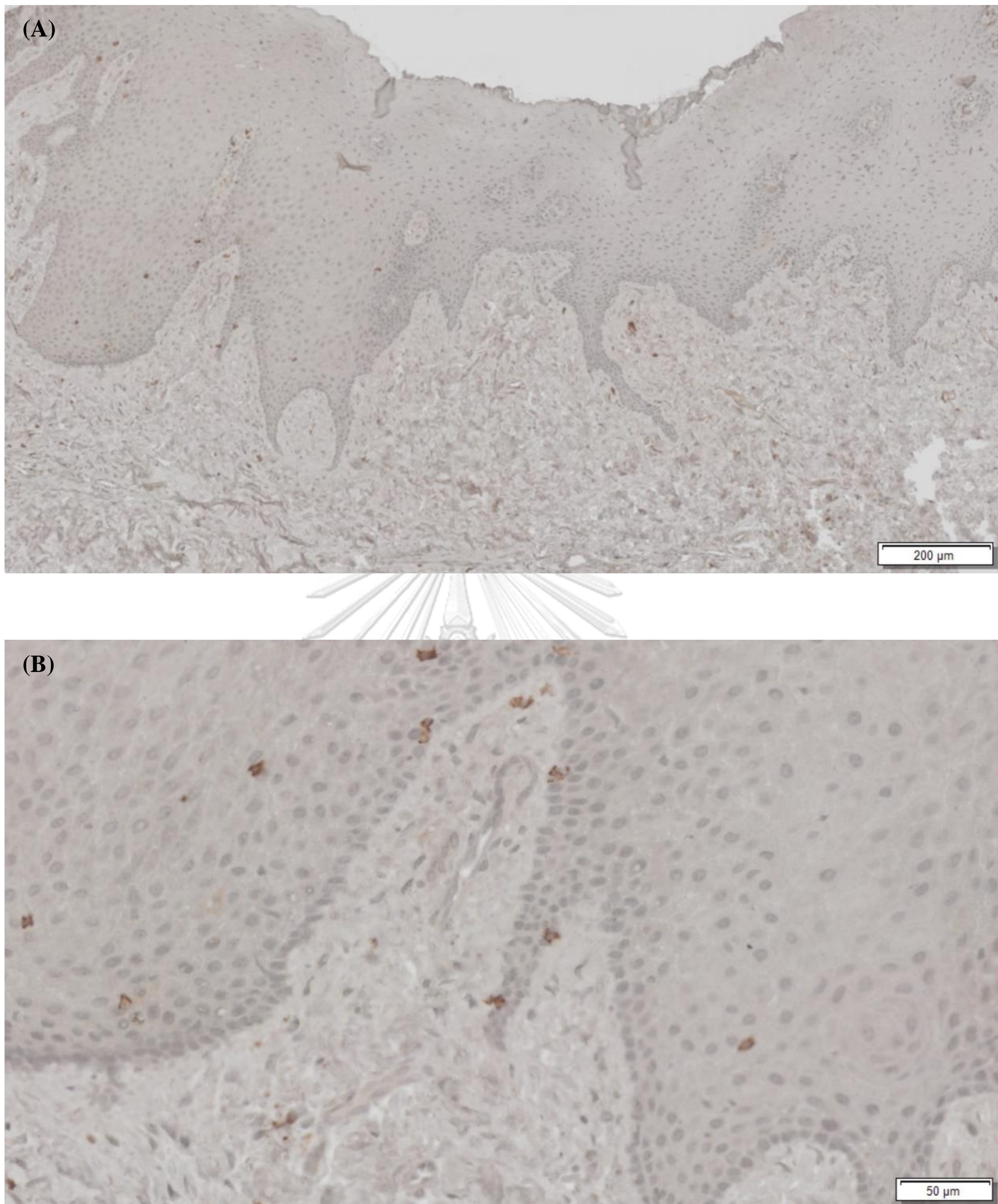

Figure 16 Immunohistochemical staining of $CD8^+$ cells in normal mucosa tissue

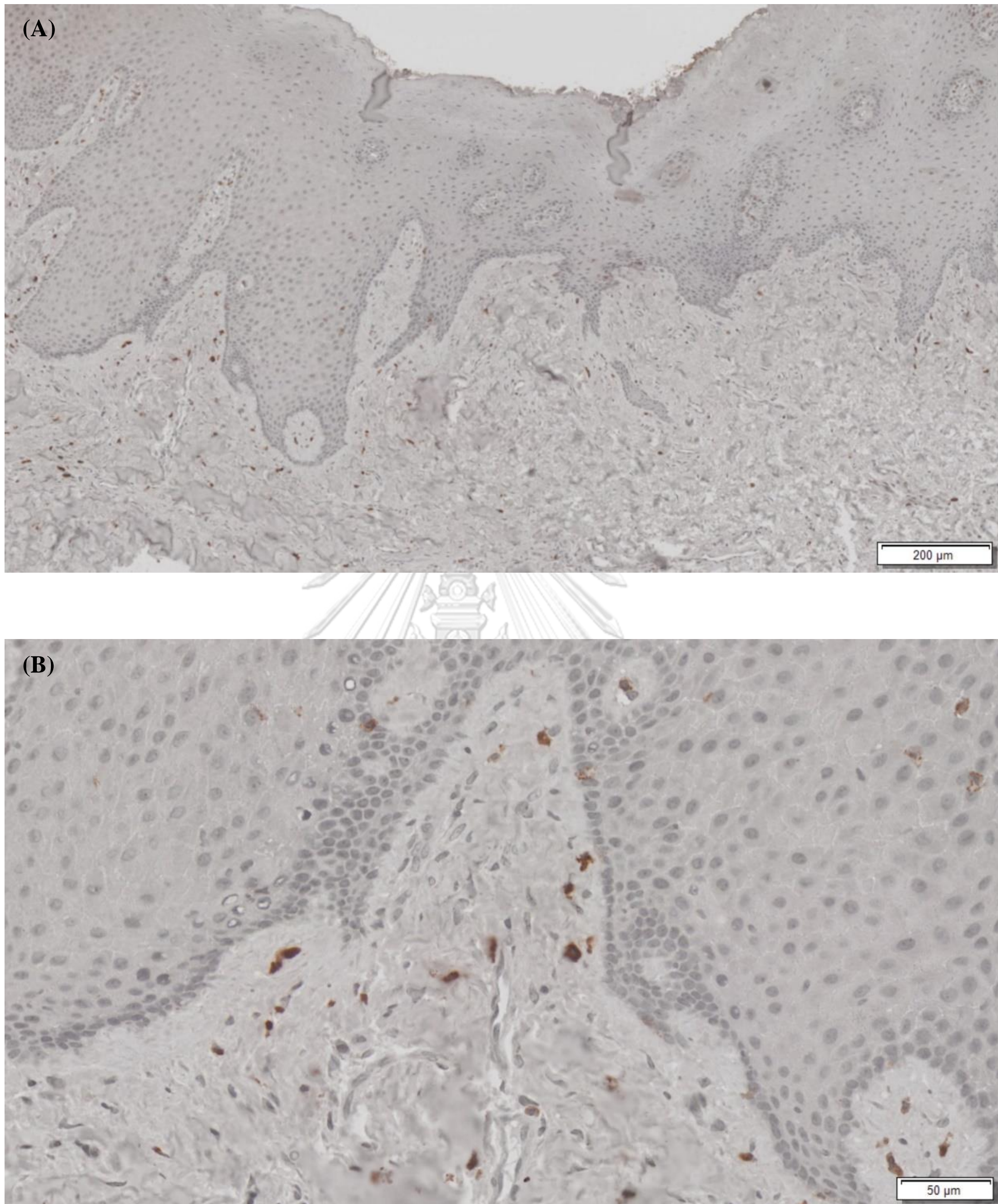

Figure 17 Immunohistochemical staining of CD103⁺ cells in normal mucosa tissue

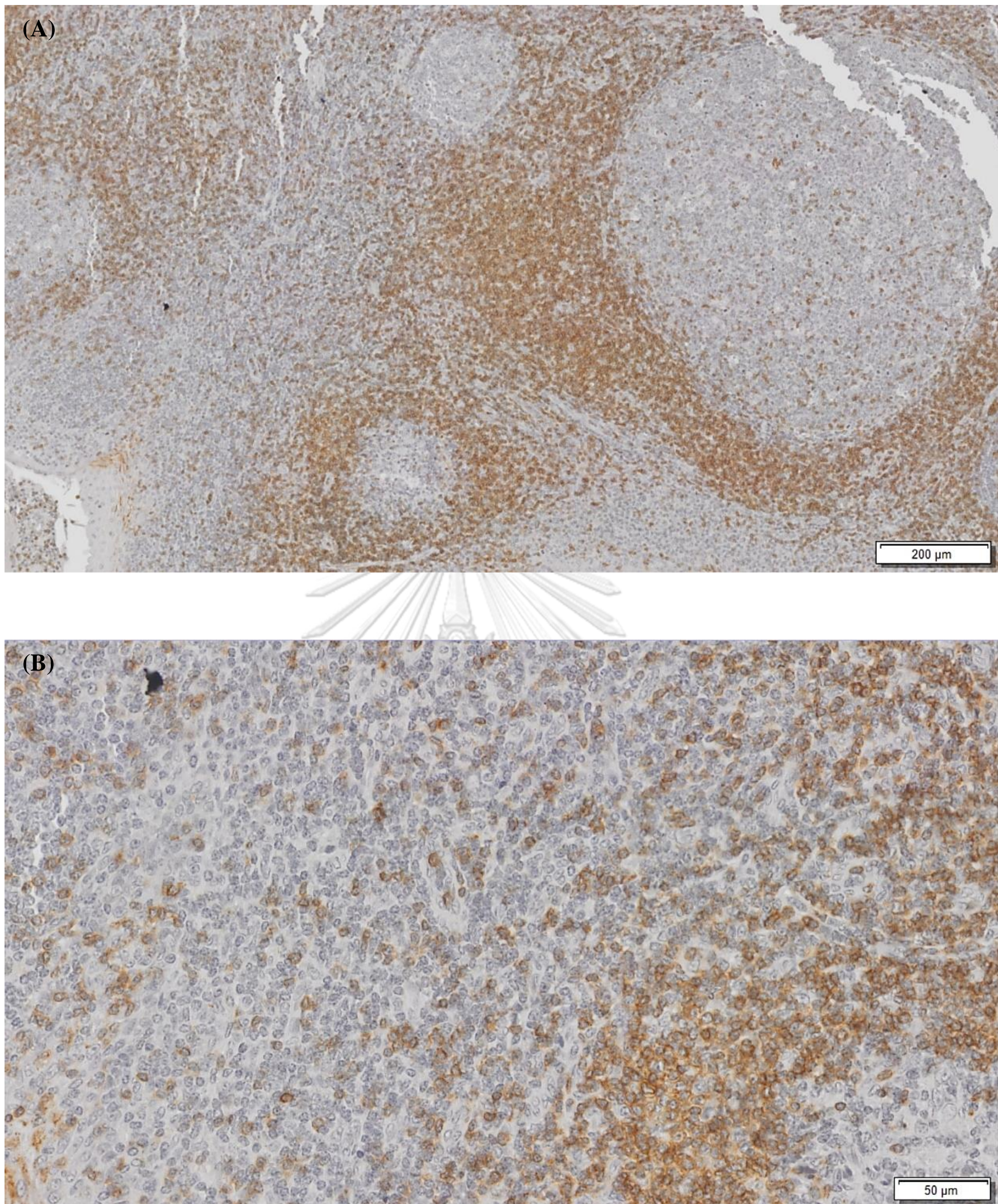

Figure 18 Immunohistochemical staining of $CD3^{+}$ cells in human tonsil tissue as a positive control

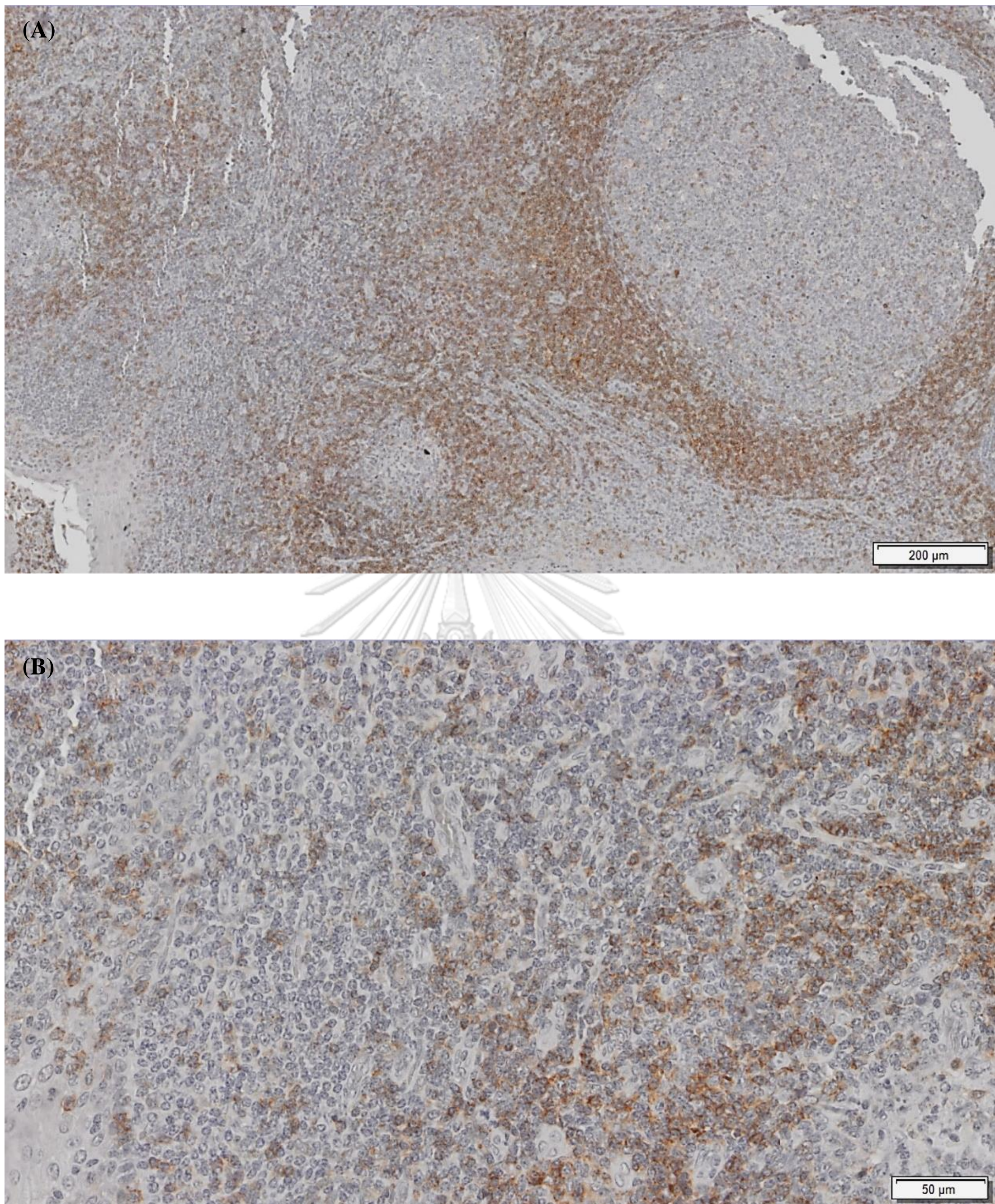

Figure 19 Immunohistochemical staining of CD4^{+} cells in human tonsil tissue as a positive control

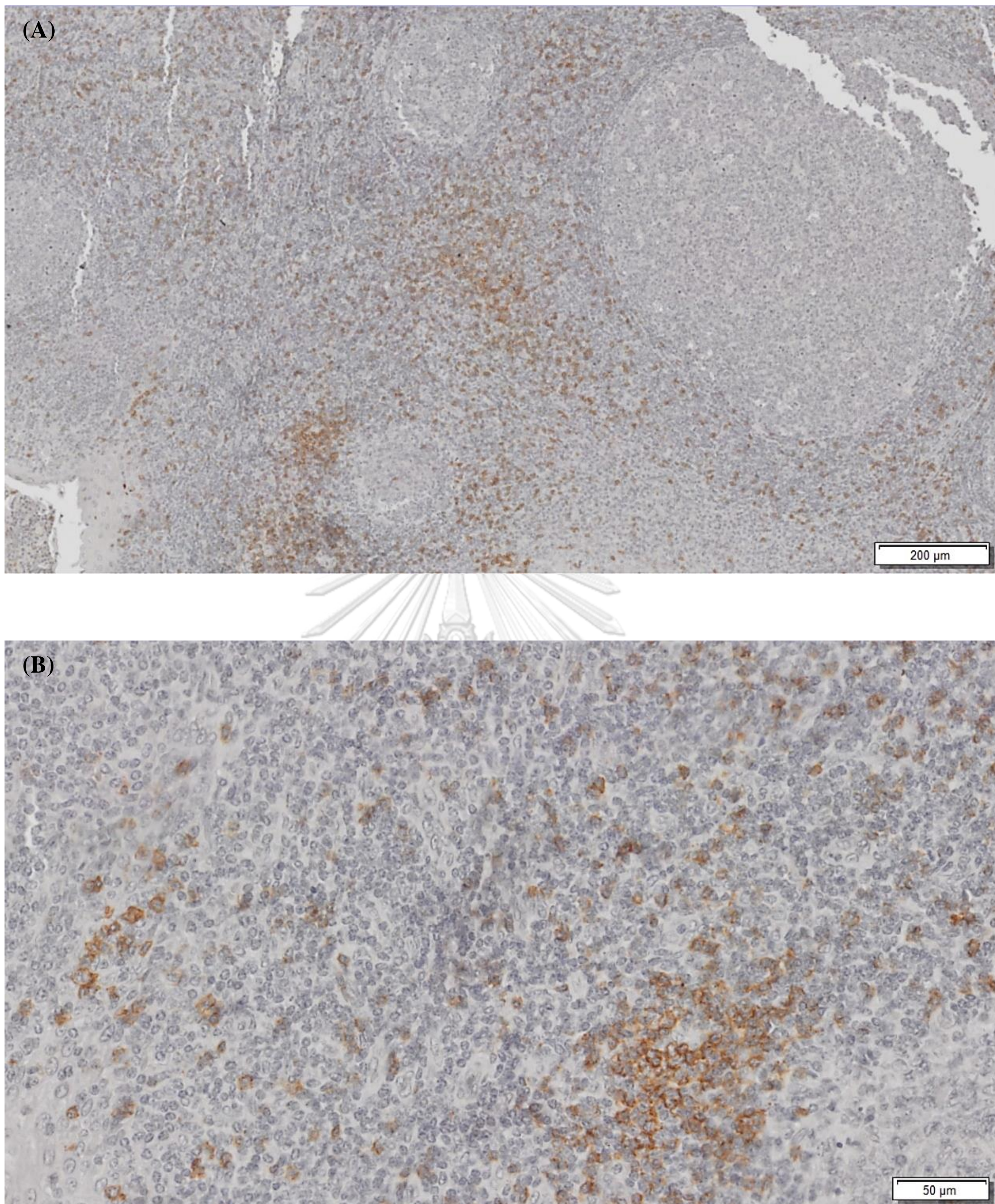

Figure 20 Immunohistochemical staining of CD8^{+} cells in human tonsil tissue as a positive control

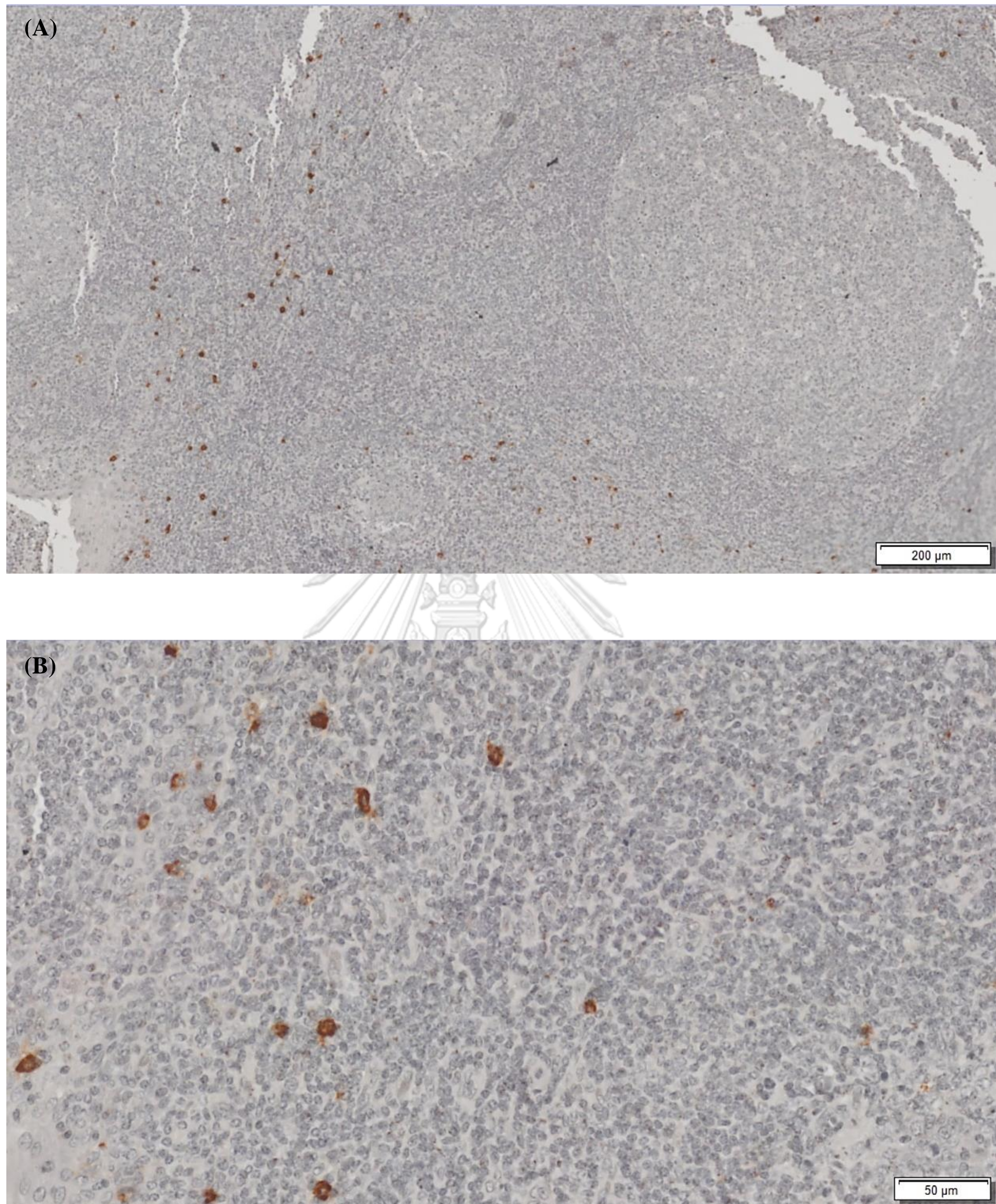

Figure 21 Immunohistochemical staining of CD103$^+$ cells in human tonsil tissue as a positive control

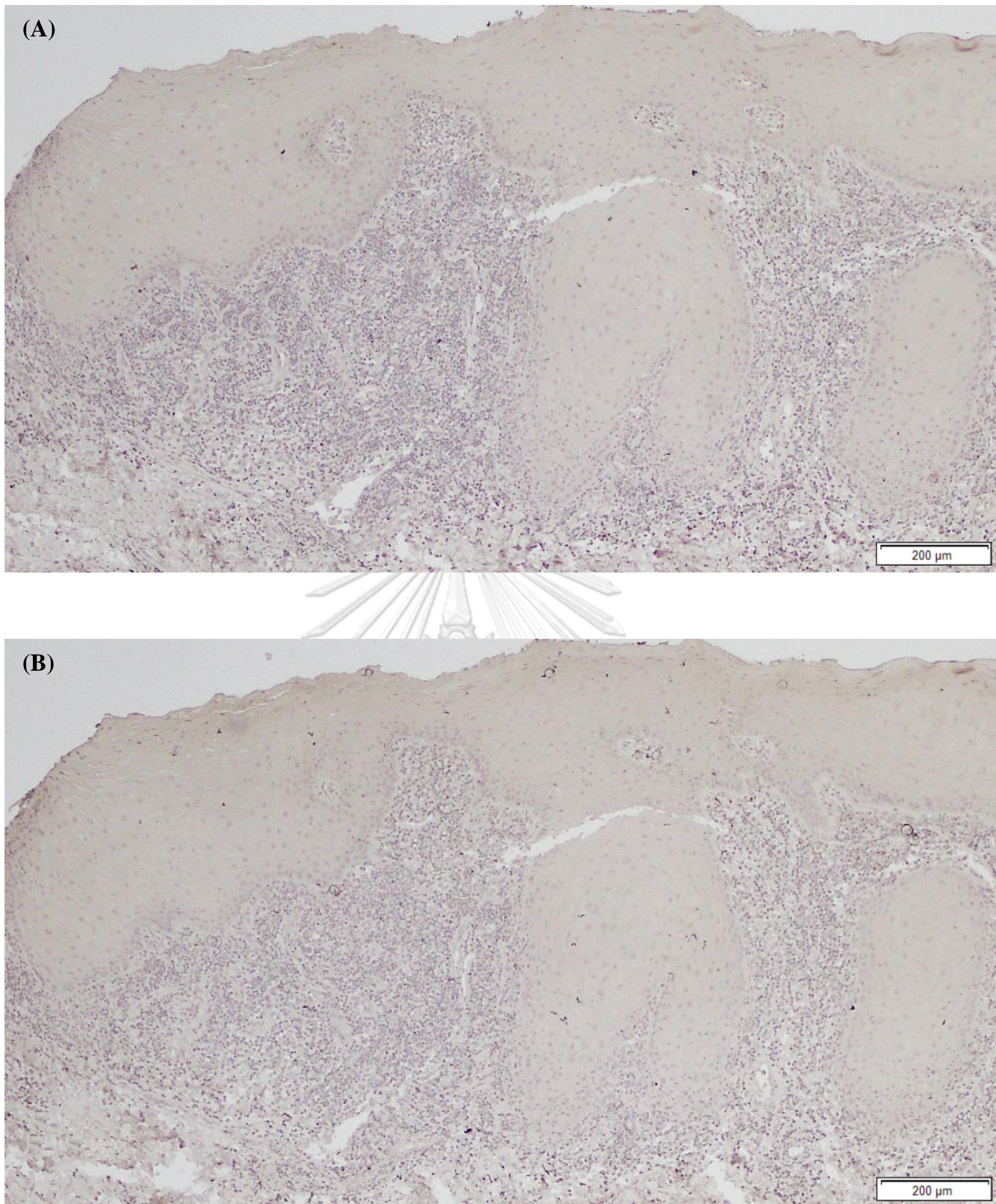

Figure 22 Immunohistochemical staining of OLP tissues as isotype controls (A) A mouse isotype control, (B) A rabbit isotype control

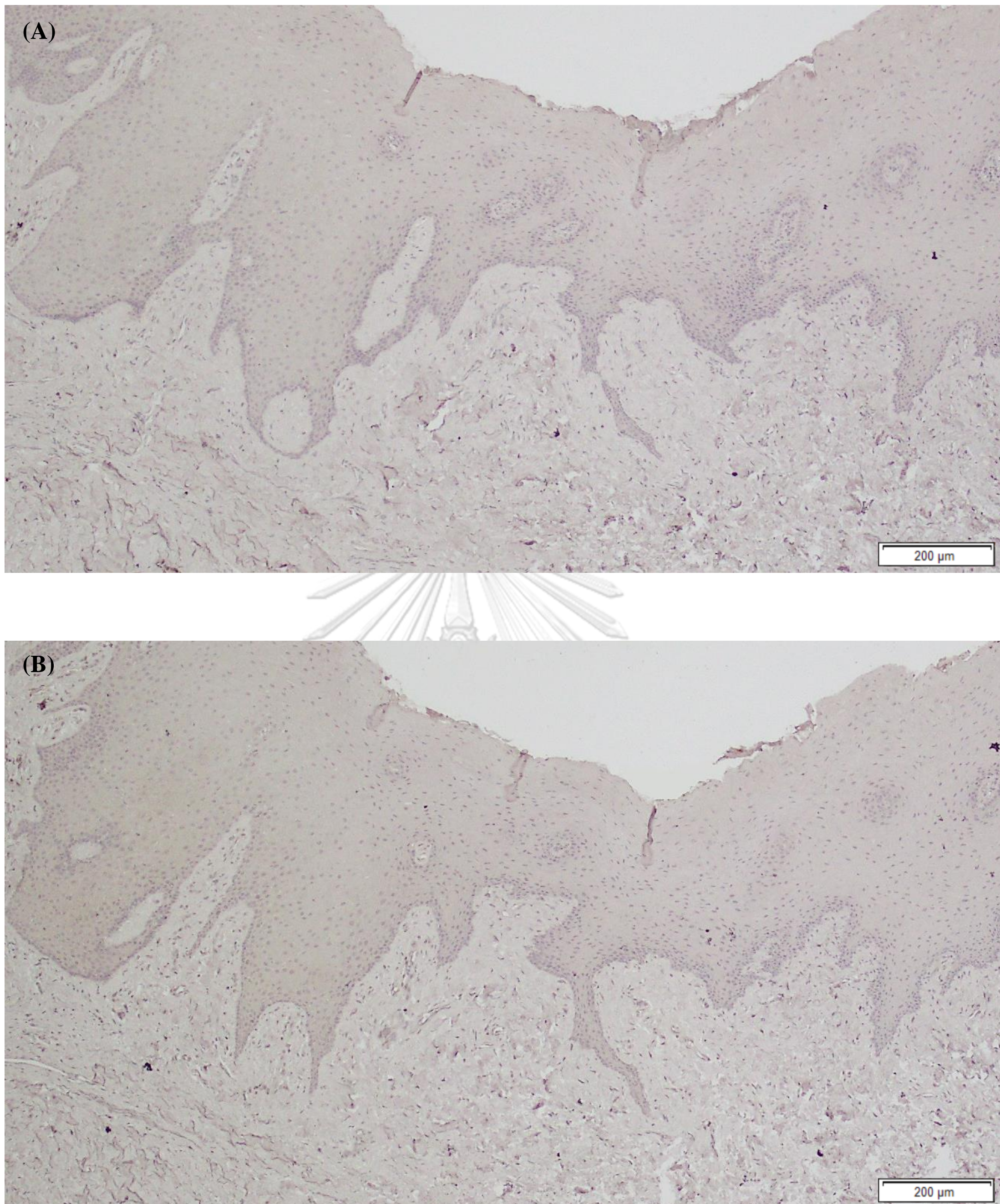

Figure 23 Immunohistochemical staining of normal mucosa tissues as isotype controls (A) A mouse isotype control, (B) A rabbit isotype control

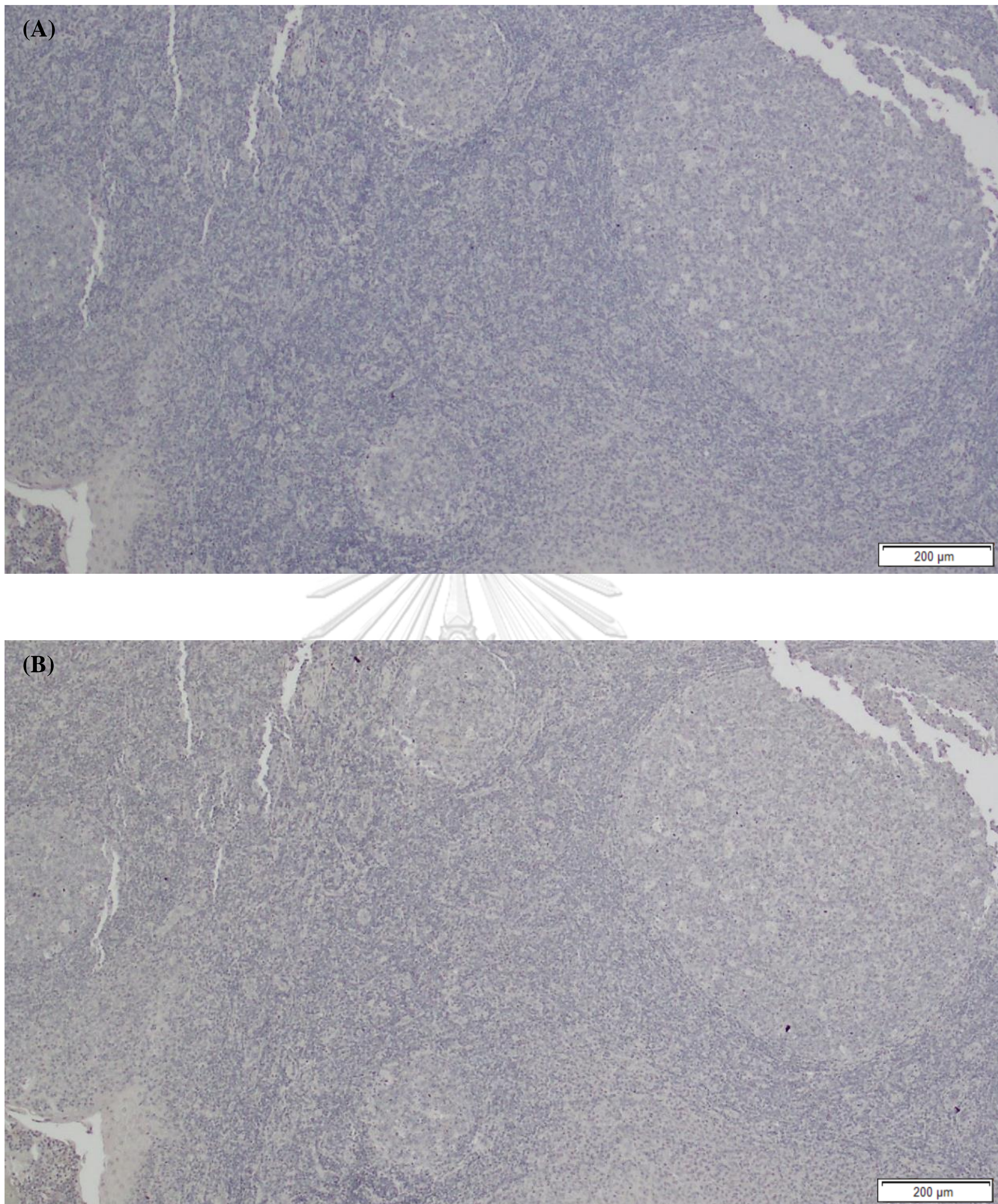

Figure 24 Immunohistochemical staining of human tonsil tissue as isotype controls (A) A mouse isotype control, (B) A rabbit isotype control

Numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area

$CD3^+$ cells were the most abundant cells found in the OLP and the normal mucosa samples. The OLP samples showed the higher number of $CD3^+$ cells per area in the epithelium (310.8 ± 234.00 cells/mm^2), in the lamina propria (7,611.8 ± 1,160.27 cells/mm^2) and in the whole sections (2,988.6 ± 913.50 cells/mm^2) when compared to the normal mucosa samples (39.7 ± 23.60, 73.3 ± 31.99 and 54.7 ± 24.62 cells/mm^2, respectively) ($p < 0.001$). The second most frequent cell in the OLP samples was $CD8^+$ cells, whereas $CD4^+$ cells were more frequent in the normal mucosa samples. However, the densities of both $CD4^+$ cells as well as $CD8^+$cells in the epithelium (CD4: 107.8 ± 84.80, CD8: 146.6 ± 95.05 cells/mm^2), in the lamina propria (CD4: 3,025.7 ± 707.52, CD8: 4,103.9 ± 875.56 cells/mm^2) and in the whole sections (CD4: 1,174.0 ± 398.04, CD8: 1,606.9 ± 584.33 cells/mm^2) of the OLP samples were still much higher than those of the normal mucosa samples (CD4: 20.0 ± 11.94, CD8: 12.6 ± 7.97 cells/mm^2; CD4: 41.0 ± 20.70, CD8: 22.0 ± 9.83 cells/mm^2; CD4 : 29.4 ± 13.90, CD8: 16.8 ± 7.52 cells/mm^2, respectively) ($p < 0.001$) *[Figure 25-27]*.

$CD3^+$, $CD4^+$ and $CD8^+$ cells were predominantly found within the lamina propria of the OLP samples. The significant differences in the densities of them between the lamina propria and the epithelium were detected ($p < 0.005$) *[Figure 28]*. The normal mucosa samples also showed higher densities of $CD3^+$, $CD4^+$ and $CD8^+$ cells in the lamina propria as compared to the epithelium ($p < 0.001$, $p < 0.001$ and $p < 0.005$, respectively) *[Figure 29]*.

$CD103^+$ cells were the less frequent cells found in this study; however, the expression of them was consistent with the other positive cells. The significant increases in the density of $CD103^+$ cells were detected in the epithelium (93.5 ± 47.82 cells/mm^2) and the lamina propria (933.1 ± 420.73 cells/mm^2) of the OLP samples compared to the normal mucosa samples (10.1 ± 5.50, 23.0 ± 13.06 cells/mm^2, respectively) ($p < 0.001$) *[Figure 25, 26]*. $CD103^+$ cells in the whole sections of the OLP samples showed about 25-fold higher density than those in the normal mucosa samples (402.3 ± 182.14 cells/mm^2 vs 16.0 ± 8.33 cells/mm^2, respectively) ($p < 0.001$) *[Figure 27]*.

In the OLP samples, the majority of $CD103^+$ cells were detected within the lamina propria which expressed approximately 10-fold higher densities than the

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epithelium ($p < 0.005$) *[Figure 28]*. In the normal mucosa samples, $CD103^+$ cells were also frequently found in the lamina propria as compared to the epithelium. The difference in the density of $CD103^+$ cells between the epithelium and the lamina propria was not much but statistically significant (about 2-fold) ($p < 0.001$) *[Figure 29]*.

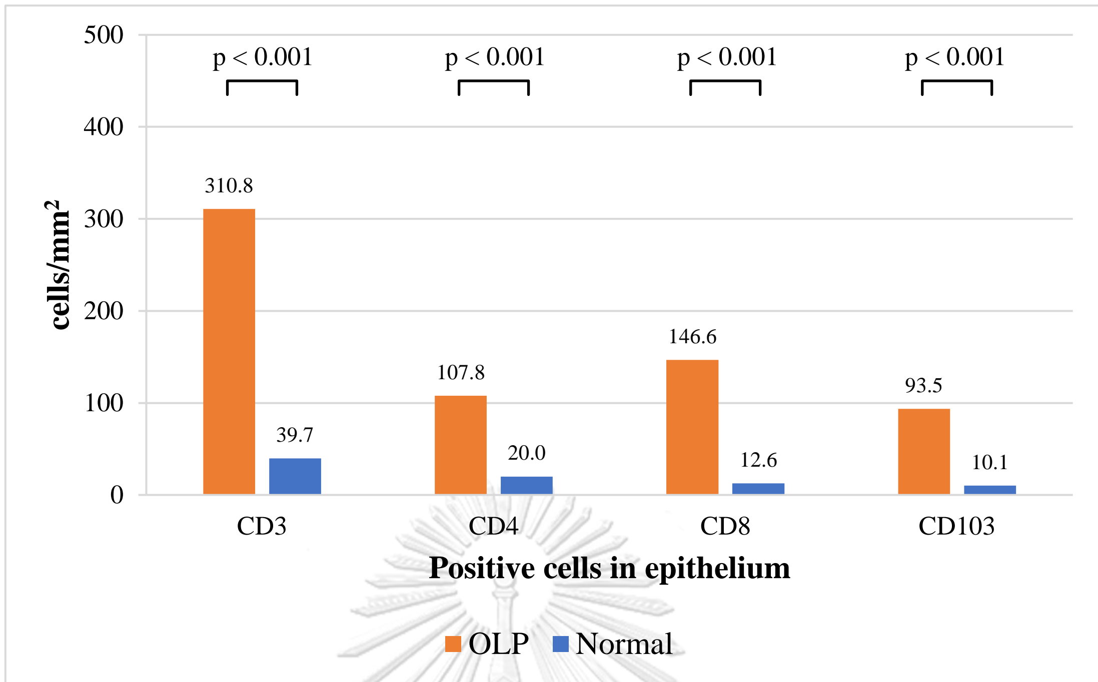

Figure 25 Numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cell/mm^2) in epithelium of OLP and normal mucosa samples

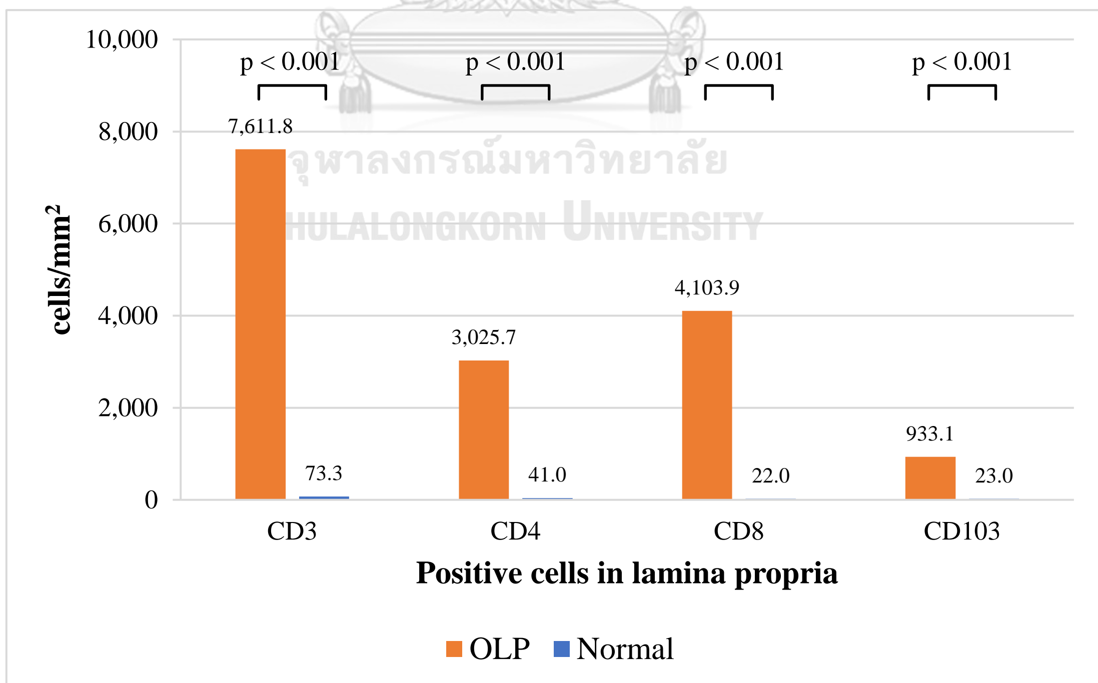

Figure 26 Numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cell/mm^2) in lamina propria of OLP and normal mucosa samples

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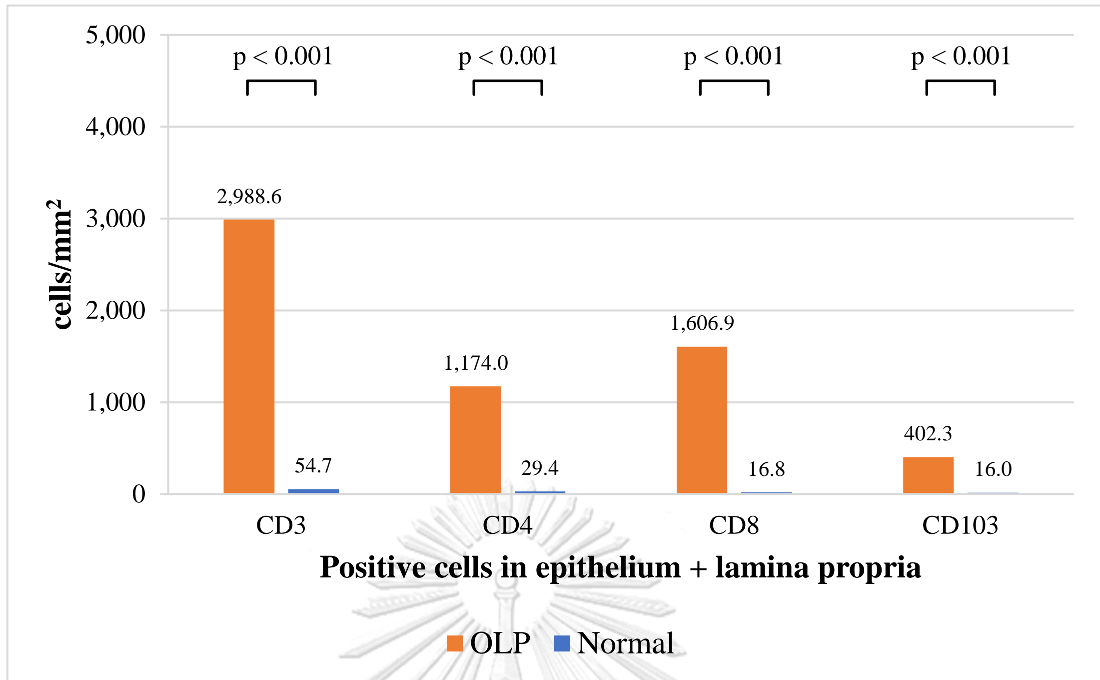

Figure 27 Numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cell/mm^2) in both epithelium and lamina propria of OLP and normal mucosa samples

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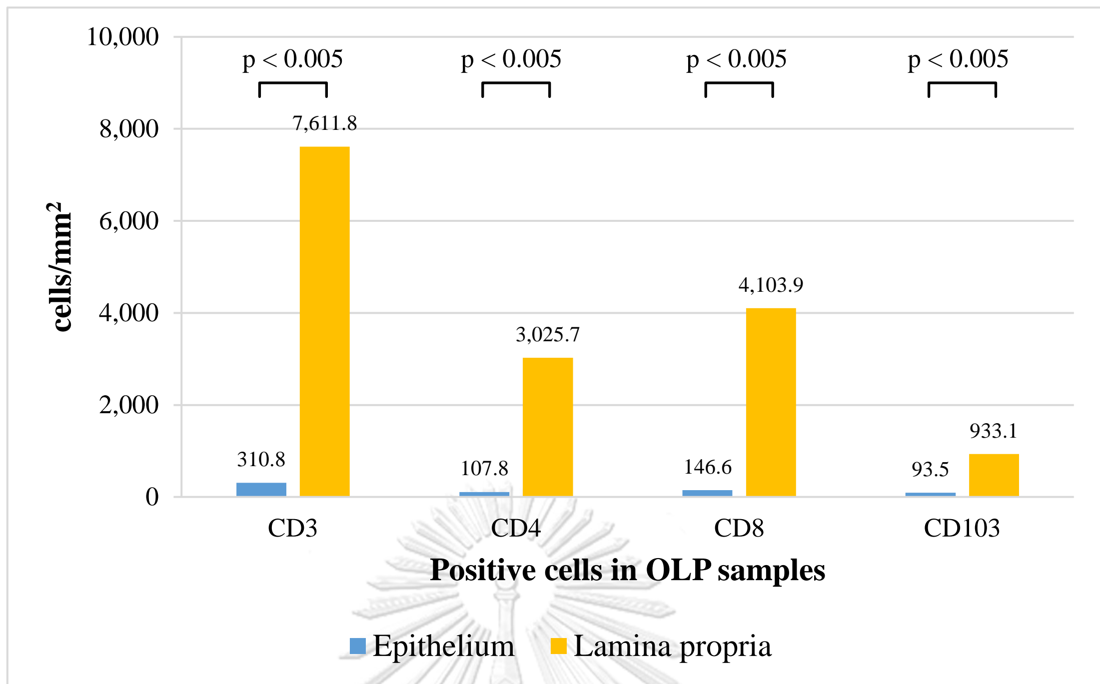

Figure 28 Numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cell/mm^2) in epithelium and lamina propria of OLP samples

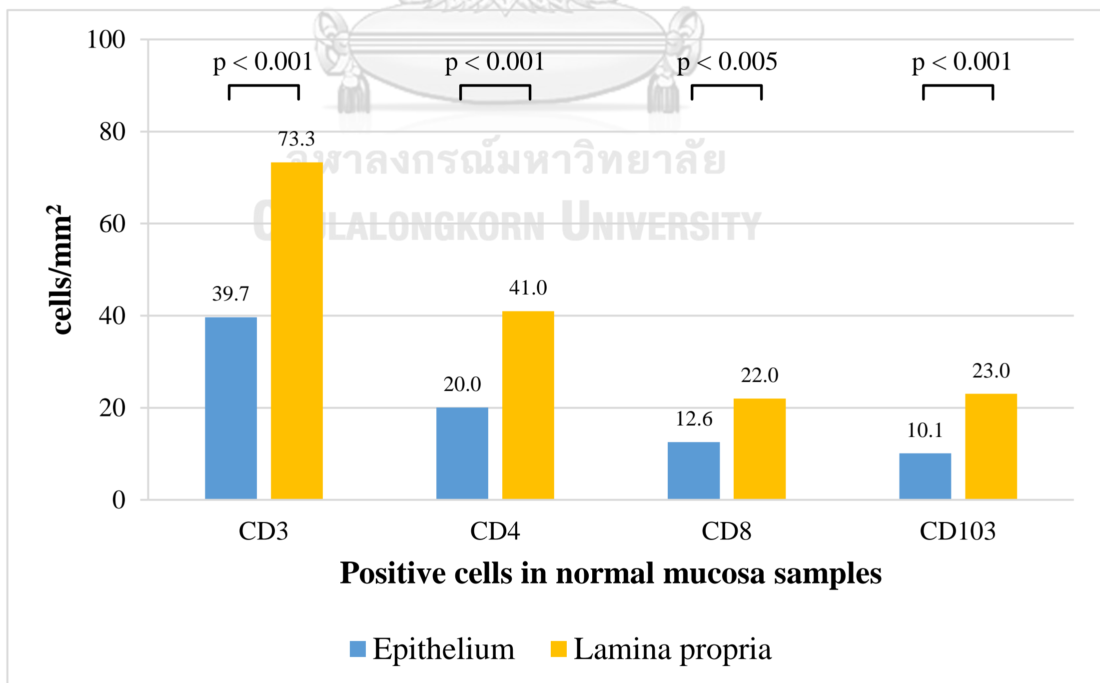

Figure 29 Numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cell/mm^2) in epithelium and lamina propria of normal mucosa samples

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Proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells

$CD8^+$ cells showed the highest proportion to $CD3^+$ cells in the OLP samples, whereas in the normal mucosa samples, $CD4^+$ cells showed the highest proportion to $CD3^+$ cells. The OLP samples expressed higher proportion of $CD8^+$ cells to $CD3^+$ cells but lower proportion of $CD4^+$ cells to $CD3^+$ cells in the epithelium (CD8: 49.3 ± 6.33, CD4: 35.3 ± 9.21 %), in the lamina propria (CD8: 53.6 ± 5.89, CD4: 39.9 ± 8.14 %) and in the whole sections (CD8: 53.2 ± 5.61, CD4: 39.6 ± 7.87 %) when compared to the normal mucosa samples (CD8: 31.9 ± 7.21, CD4: 50.9 ± 8.12 %; CD8: 30.6 ± 7.26, CD4: 54.5 ± 5.1 %; CD8: 31.2 ± 5.40, CD4: 53.3 ± 5.88 %, respectively) ($p < 0.001$) *[Figure 30-32]*. The proportion of $CD8^+$ cells to $CD3^+$ cells was about 1.3-fold higher than the proportion of $CD4^+$ cells to $CD3^+$ cells in the OLP samples.

$CD103^+$ cells showed the lowest proportion to $CD3^+$ cells in both the OLP and the normal mucosa samples. In the epithelium, the proportion of $CD103^+$ cells to $CD3^+$ cells in OLP samples was higher than that in the normal mucosa samples; however, this difference was not statistically significant (34.7 ± 10.62 % vs 27.6 ± 9.53 %, respectively) ($p = 0.062$) *[Figure 30]*. On the contrary, the lamina propria of the OLP samples expressed significantly lower proportion of $CD103^+$ cells to $CD3^+$ cells as compared to the normal mucosa samples (12.2 ± 5.06 % vs 31.0 ± 12.63 %, respectively) ($p < 0.001$) *[Figure 31]*. The whole sections of the OLP samples also expressed approximately 2-fold lower proportion of $CD103^+$ cells to $CD3^+$ cells than the normal mucosa samples (13.6 ± 4.97 % vs 29.3 ± 9.76 %, respectively) ($p < 0.001$) *[Figure 32]*.

In the OLP samples, the proportions of $CD4^+$ and $CD8^+$ cells to $CD3^+$ cells in the epithelium were comparable to those in the lamina propria ($p = 0.148$ and $p = 0.089$, respectively), but the proportion of $CD103^+$ cells to $CD3^+$ cells in the epithelium increased about 3-fold as compared to that in the lamina propria ($p < 0.001$) *[Figure 33]*. In the normal mucosa samples, there were no statistical differences in the proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells between the epithelium and the lamina propria ($p = 0.056$, $p = 0.615$ and $p = 0.346$, respectively) *[Figure 34]*.

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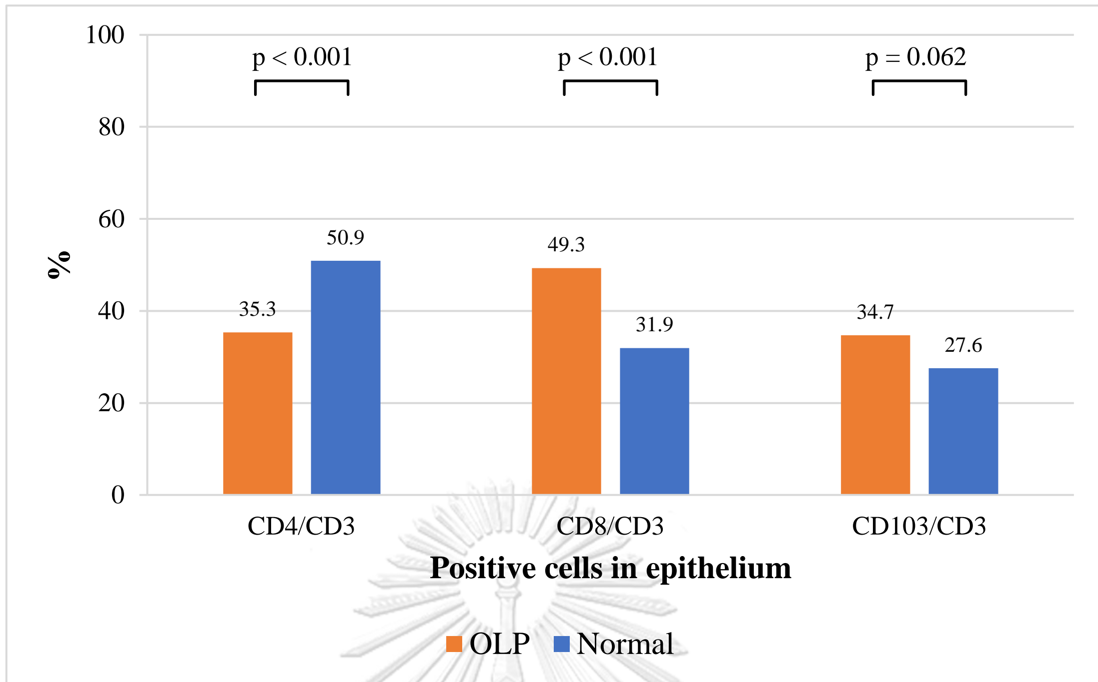

Figure 30 Proportion of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) in epithelium of OLP and normal mucosa samples

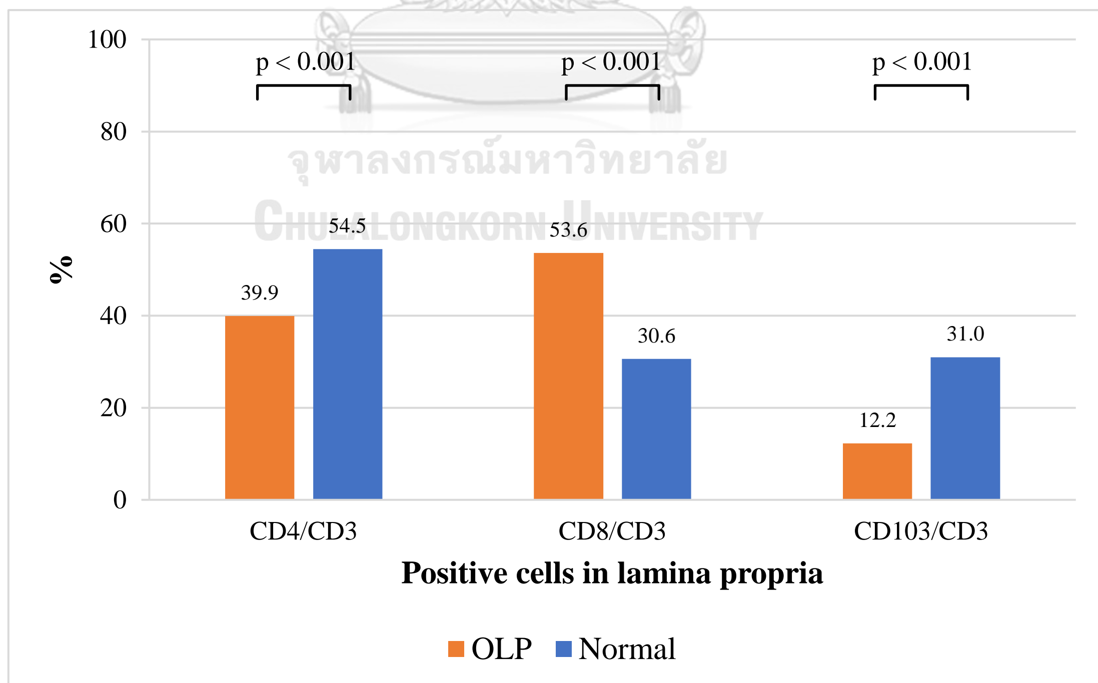

Figure 31 Proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) in lamina propria of OLP and normal mucosa samples

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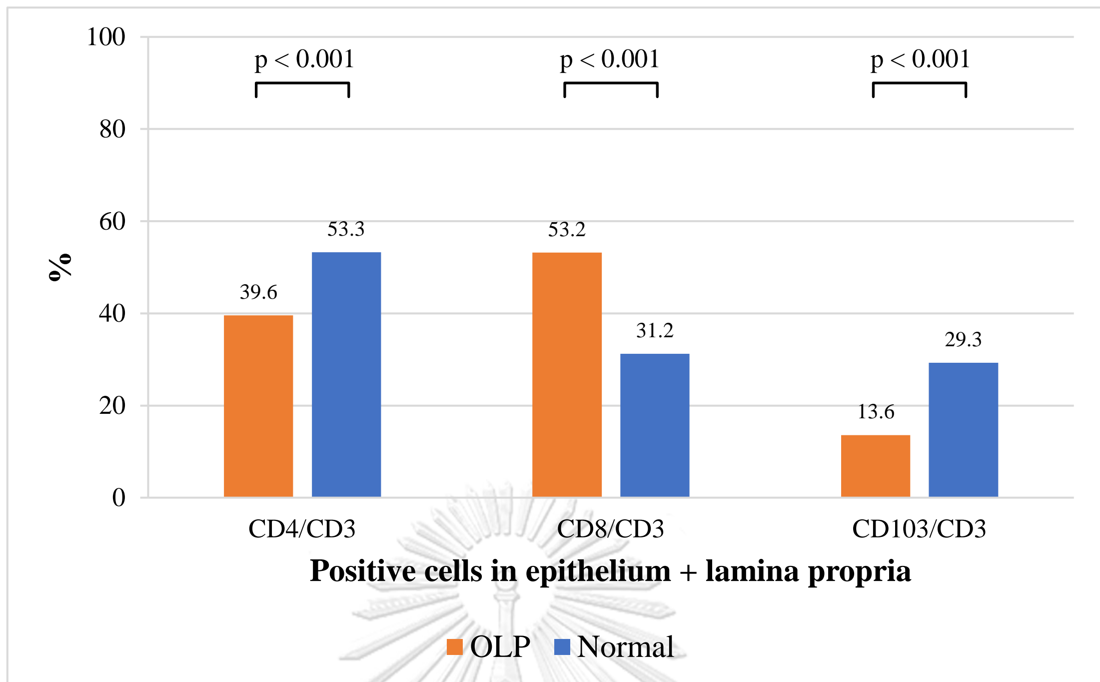

Figure 32 Proportions of CD4^{+}, CD8^{+} and CD103^{+} cells to CD3^{+} cells (%) in both epithelium and lamina propria of OLP and normal mucosa samples

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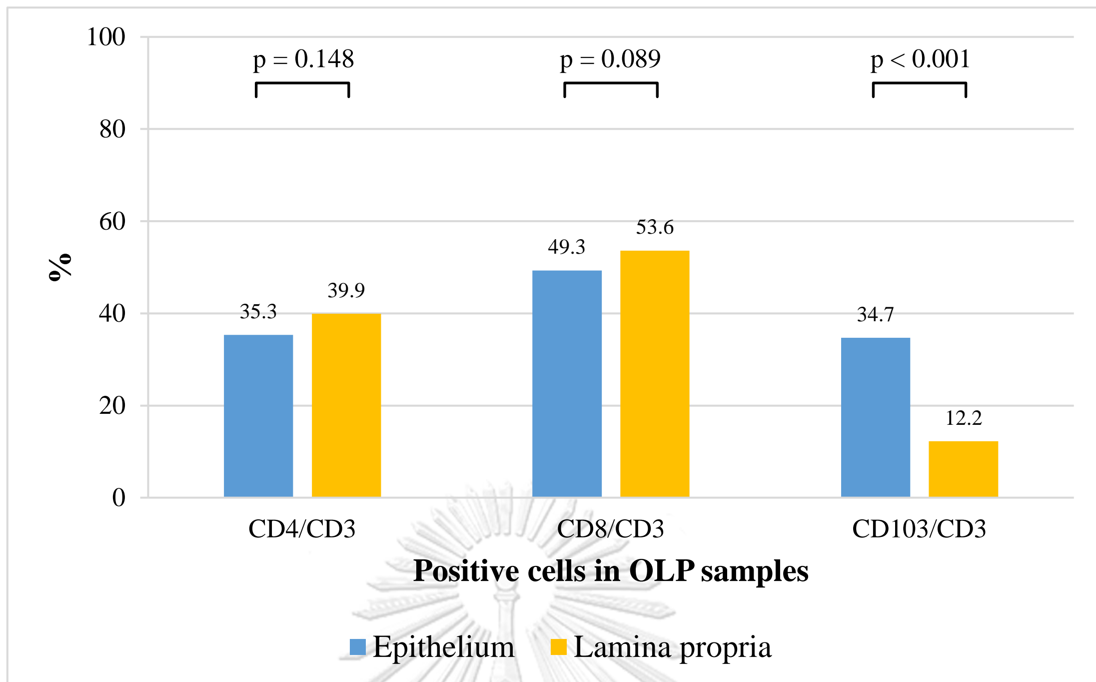

Figure 33 Proportions of CD4$^+$, CD8$^+$ and CD103$^+$ cells to CD3$^+$ cells (%) in epithelium and lamina propria of OLP samples

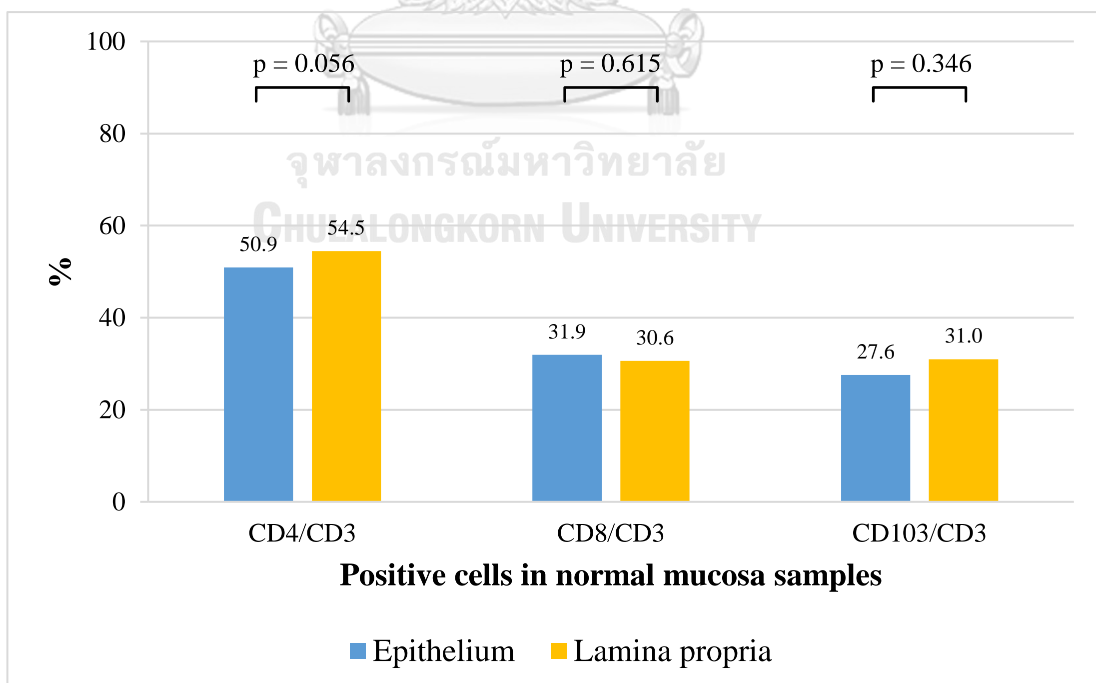

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Figure 34 Proportions of CD4$^+$, CD8$^+$ and CD103$^+$ cells to CD3$^+$ cells (%) in epithelium and lamina propria of normal mucosa samples

CHAPTER V

DISCUSSION

It is known that OLP is predominantly infiltrated with T cells which are mostly represented by $CD8^+$ T cells rather than $CD4^+$ T cells[8, 45]. In this study, the results were consistent with several studies showing the marked increases in the densities of $CD3^+$, $CD4^+$ and $CD8^+$ cells in the OLP lesions as compared to those in the normal mucosa *[Figure 27]*. The proportion of $CD8^+$ cells in OLP was also higher than the proportion of $CD4^+$ cells with the ratio of $CD8^+$ cells:$CD4^+$ cells approximately of 1.3:1 *[Figure 32]*. Similar to this study, Villarroel Dorrego et al. reported that OLP showed the significantly higher numbers of $CD4^+$ cells and $CD8^+$ cells than the normal mucosa, and $CD8^+$ cells represented the majority of infiltrating T cells in OLP[49]. The ratio of $CD8^+$ cells:$CD4^+$ cells in OLP calculated from several studies ranged between 1-2:1[47, 49, 116, 117].

This study also agreed with the previous study in the sense that OLP generally expressed higher proportion of $CD8^+$ cells, but normal mucosa tended to express higher proportion of $CD4^+$ cell[47] *[Figure 32]*. However, a few OLP specimens in this study expressed $CD4^+$ cells more than $CD8^+$ cells. Some studies found the high proportion of $CD4^+$ T cells in OLP as well[46, 118]. This variation may be due to progression of the OLP disease. Early OLP lesions prone to present normal characteristics than long-lasting OLP lesions; thus, $CD4^+$ T cells constitute a higher proportion of infiltrating T cells. As the lesions were chronic, these $CD4^+$ T cells together with other intra-lesional cells may promote the influx of numerous $CD8^+$ T cell; therefore, $CD8^+$ T cells become the principal cells in the lesions[49].

In OLP, T cells were abundant in the lymphocytic bands of the lamina propria. Khan et al. stated that the majority of T cells in the OLP epithelium and the OLP superficial lamina propria were $CD8^+$ T cells, while most of T cells in the OLP deep lamina propria were $CD4^+$ T cells[50]. In contrast, this study detected no significant difference in the proportion of $CD4^+$ and $CD8^+$ cells between the epithelium and the lamina propria of OLP *[Figure 33]*. Stages of disease and degrees of basement

membrane disruption may have an impact on this contrast. $CD8^+$ T cells migrate into the OLP epithelium through areas of basement membrane breakdown, while $CD4^+$ T cells do not exhibit this selective migration[51]. Thereby, the more severe the lesion appears, the greater number of intreaepithelial $CD8^+$ T cells may be observed. The study of Zhou et al. has demonstrated an increase in the number of $CD8^+$ T cells, but not $CD4^+$ T cells within the OLP epithelium at regions of membrane disruption when compared to regions of basement membrane continuity. They speculated that $CD8^+$ T cells may express some adhesion molecules to support this migration process[51].

In the OLP epithelium, this study revealed that $CD3^+$, $CD4^+$ and $CD8^+$ cells were often found adjacent to or within the degenerative epithelial cell layers in which $CD8^+$ cells were slightly more pronounced than $CD4^+$ cells. These findings were in lined with the study by Khan et al[50]. However, in the OLP lamina propria, the study by Khan et al. pointed out dominant distribution of $CD8^+$ cells at the superficial parts and $CD4^+$ cells at the deep parts[50] which contrasted with this study that presented intermixing locations of $CD4^+$ and $CD8^+$ cells in the lymphocytic bands without grouping of exclusive cell types. Despite this discrepancy, all the above-mentioned evidence considerably supported the involvement of T cells in the pathogenesis of OLP. Notably, most of the OLP sections showed the thin space layers without $CD3^+$, $CD4^+$ and $CD8^+$ cells beneath epithelium-lamina propria interfaces. These spaces were supposed to be occupied by APCs, such as Langerhans cells, myeloid dendritic cells and PDCs, in order to deliver peptide/MHC complex to T cells.

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Regarding $CD103^+$ cells, this study found a 25-fold increase in the density of $CD103^+$ cell in OLP compared to the normal mucosa *[Figure 27]*. However, when turn this into a proportion to $CD3^+$ cells, normal mucosa expressed higher proportion of $CD103^+$ cells than OLP *[Figure 32]*. Furthermore, this study also found that, in OLP, the proportion of $CD103^+$ cells in the epithelium was about 2-fold higher than that in the lamina propria *[Figure 33]*. It was because OLP contained extremely large number of $CD3^+$ cells particularly within the lymphocytic bands, so concentration of $CD103^+$ cells in OLP especially within the lamina propria was diluted. This study confirmed the findings of Walton et al. in 1997 which was the only one study regarding $CD103^+$ T_{RM} cells in OLP. The study by Walton et al. also showed a significant increase in the

expression of $CD103^+$ cells in OLP, and a higher proportion of $CD103^+$ cells to $CD3^+$ cells in the OLP epithelium as compared to the OLP lamina propria (about 6-fold). Most of $CD103^+$ cells were present in the basal epithelial layers of OLP, resembling the distribution fashion exhibited in the current study. Moreover, they investigated the expression of $CD103^+$ T_{RM} cells in the peripheral blood and stated that there was no significant difference in the number of $CD103^+$ T_{RM} cells between the OLP patients and the normal healthy subjects[17]. However, the study by Walton et al. neither investigated the expression of $CD3^+$, $CD4^+$ and $CD8^+$ cells nor emphasized the distribution patterns of $CD103^+$ cells within the epithelium and the lamina propria of the OLP tissues. They focused on the adhesion property of CD103 for T cell localization rather than the immune actions of T cells expressing CD103.

Although this study and the study by Walton et al. gave the results at the same pace, the proportion of $CD103^+$ cells from this study was about 2-3-fold lower than those from the other previous study. This difference may be due to several factors. Regarding the OLP specimens, both of the studies obtained the samples from the buccal mucosa but included different OLP types. The ulcerative/erosive OLP was the most frequent OLP type in this study, whereas the other study chose only the reticular OLP. Since $CD103^+$ cells are the long-persistent cells in the oral mucosa[14] and $CD3^+$ T cells prone to largely infiltrate into the erosive OLP rather than the reticular OLP[116]. Therefore, the OLP specimens in this study, mainly ulcerative/erosive type, might contain a larger number of $CD3^+$ cells that make a ratio of $CD103^+$ cells per $CD3^+$ cells accordingly reduced. Furthermore, a difference in disease durations might result in varying disease stages that could influence on the composition of the inflammatory cell pool as well.

Owing to ethical limitation, the normal mucosa tissues used in this study were taken from the non-inflammatory mucosa of patients undergoing impacted tooth removal (regarded as buccal flaps) as an alternative to the buccal mucosa of healthy volunteers that was used in the study of Walton el al. A buccal flap is commonly advanced from gingiva. In this study, the gingiva was the second most common OLP affected site following the buccal mucosa. However, gingiva is characterized by keratinized stratified squamous epithelium, but buccal mucosa is composed of non-

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keratinized stratified squamous epithelium[119]. A keratinized layer is an important physical barrier in an innate immunity protecting underlying tissues from pathogen penetration; thus, the keratinized tissues may have lower chances of infection and inflammation than the non-keratinized tissues[55]. However, several studies on the OLP immunopathogenesis also used the oral mucosa from the sites of impacted tooth removal to be the normal mucosa samples as same as this study[120, 121].

All the samples in this study were prepared from the paraffin-embedded tissues, while Walton et al. utilized frozen tissue sections in their study. Some studies claimed that due to the better preservation of antigen contents, the frozen tissue sections are more sensitive for detection of antigen-antibody binding activity than the paraffin-embedded tissue sections[122, 123]. However, more recent studies debated that the paraffin-embedded tissues with an antigen retrieval treatment, the technique performed in this study, showed comparable immunohistochemical staining results to the frozen tissues[124, 125]. Thereby, the difference in the tissue processing might not yield a significant consequence to the study.

Although the recommended section thickness for immunohistochemistry is generally 4 µm[126, 127], in this study, the sections were cut as thin as 2 µm in thickness. Because a problem was found that the 3-4-µm-thick sections having been cut before usually detached from glass slides during the process. According to the study of Gambella et al. that demonstrated a linear increase in the section detachment with increasing the section thickness[128], this study thus decreased the section thickness to 2 µm which was the maximum thickness with minimal detachment. Note that this study used thinner section thickness than the study of Walton et al. which prepared in 5-µm thickness. McCampbell et al. reported influence of the section thickness on cell expression that a 2 µm difference in thickness cut could affect staining intensity to the degree that cells might be incorrectly identified as positive or negative stained cells[129]. A variation in the cell quantity between this study and the study by Walton et al. might partially result from this factor.

One of the important determinants influencing cell expression and cell detection is the primary antibody clone. To detect $CD103^+$ cells by means of single-labelling immunohistochemistry, rabbit anti-human CD103 monoclonal antibody, clone EPR4166(2) - IgG was used in this study, whereas in the previous study, Walton et al.

used mouse anti-human CD103 monoclonal antibody, clone 2G5.1 - IgG2a[17]. The different clones of the monoclonal antibodies specifically bind the different epitopes of the same target antigen due to their variation in antibody sensitivities and specificities; therefore, quality and quantity of the signals generated by the different primary antibody clones may be dissimilar[130].

In addition, the immunohistochemical staining method between these 2 studies were also different. This study used a labelled polymer method of which visualization based on HRP-labelled polymer with DAB+ substrate-chromogen. The other study used a labelled streptavidin-biotin method that employed alkaline phosphatase as an enzyme paired with naphthol AS-Bl phosphate and new fuchsin as a chromogenic dye[17, 131]. Because the labelled polymer method allows large numbers of antibodies and enzymes to conjugate with a polymer backbone, this system provides much better detection sensitivity compared to the conventional labelled streptavidin-biotin system[132, 133].

Remarkably, in the current study, the healthy normal subjects were younger than the OLP patients because there was the ethical limitation and biopsy of the normal mucosa from age-matched subjects without a medical indication was restricted. However, to the best of my knowledge, no study claimed the quantity of $CD103^+$ T_{RM} cells was age-dependent, so that age might not cause a marked impact on this study's results.

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Since the tissue samples varied in sizes and this might affect the amount of the infiltrating cells, the results were presented as the density of the positive cells (the number of the positive cells per area) instead of the total amount of them. This study also examined the expression of $CD3^+$, $CD4^+$ and $CD8^+$ cells besides $CD103^+$ cells and presented the percentage data of $CD103^+$ cell to $CD3^+$ cells in order to elucidate the patterns of expression and localization among these cells.

The total area of the epithelium was simple to calculate due to its own clearly defined boundary. The lamina propria always showed ragged outline at the bottommost part that made it complicated to determine the definite area. In OLP, the area of lymphocytic bands was assigned to be a representative area of the lamina propria since almost all of the positive cells in the lamina propria localized within this band.

However, in the lamina propria of the normal mucosa, there was no apparent evidence that could be used to specify the calculated area; therefore, the average width of the OLP lymphocytic bands was designated as a width of the lamina propria for all of the normal mucosa samples. Most of the positive cells in the lamina propria of the normal mucosa were detected within this defined area as well.

T_{RM} cells occupy a wide range of tissues[14]. In the oral cavity, to the best of my knowledge, their existence was substantially mentioned in 3 articles[17, 84, 85]. The first one reported that $CD103^+$ T_{RM} cells examined by means of double-labelling immunohistochemistry were largely observed in junctional epithelium and oral gingival epithelium than the underlying connective tissues of both healthy and periodontitis gingiva[84]. The second one, an aforementioned study by Walton et al., pointed out the higher percentage of $CD103^+$ cells to $CD3^+$ cells in the OLP epithelium when compare to that in the OLP lamina propria[17]. The last one, a recent study in the Thai subjects, also found $CD103^+$ cells in the epithelium and the lamina propria of the periodontitis tissues upon single-labelling immunohistochemical staining[85]. In this study, the OLP epithelium expressed the higher proportion of $CD103^+$ cells to $CD3^+$ cells than the OLP lamina propria as well.

It can be seen that $CD103^+$ T_{RM} cells localize within both the epithelium and the lamina propria of the oral mucosa. CD103 molecules help promote adherence of T_{RM} cells in the epithelium by interacting with E-cadherins which are the adhesion molecules specifically expressed by the epithelial cells[99], so that $CD103^+$ T_{RM} cells were considerably observed within the epithelial layer in preference to the underlying lamina propria. The existence of $CD103^+$ T_{RM} cells in the subepithelial regions indicates whether other adherence manners besides CD103 activity are required for the maintenance of $CD103^+$ T_{RM} cells. There is the evidence that T_{RM} cells employ a cooperative combination of CD103, CD49a, CD44 and CD69 to retain them within the previous inflammation sites. CD49a or $\alpha1\beta1$ integrin interacts with collagens preferably collagen type IV in the basement membrane. CD44 binds a wide variety of tissue elements including hyaluronic acid, fibronectin and other extracellular matrix proteins in the lamina propria. CD69 antagonizes S1PR1 functions and then inhibits emigration from the peripheral tissues[134]. Additionally, T_{RM} cells can express E-

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cadherins by themselves leading to homotypic interaction within their population and with other E-cadherin-expressing cells such as epithelial cells and antigen presenting cells. This tethering may advocate not only cell maintenance in the peripheral tissue but also cell-cell communication with stable synapses[134, 135]. In addition to the physical adherence, CD103 are alternatively hypothesized to help promote survival of T_{RM} cells[13, 135].

Contrary to CD103, the expression of E-cadherin on the epithelial cells decreases in OLP as compared to the normal mucosa. E-cadherin expression is lost focally in actively diseased areas of the basal epithelial cell layer but is conspicuous in non-diseased areas. This loss of E-cadherin expression may contribute to basal epithelial cell degeneration and T cell migration into the OLP epithelium[107-109, 136].

Regarding the other adhesion molecules on T_{RM} cells, information about the expression of CD69, CD49a and CD44 in OLP was not much available. Either increases or decreases in the expression of these adhesion molecules in OLP were documented. Some studies stated no significant difference in their expression between OLP and the normal mucosa. Therefore, it was ambiguous to interpret whether these molecules actually assisted or inhibited the localization and the functions of T_{RM} cells in OLP. However, it is remarkable that most studies reported the pronounced expression of these adhesion molecules in the basal and the parabasal cell layers of OLP[104-106, 109, 111, 137-141]. Furthermore, the available studies on epithelial cell-derived TGF-β, which is the cytokine for T_{RM} cell maintenance, reported controversial results about the expression of this cytokine in OLP as well[112-114].

In this study, the amount of $CD103^{+}$ cells increased in OLP over the normal mucosa. This accumulation may due to the differentiation of the immigrating T cells into $CD103^{+}$ T_{RM} cells as well as the local proliferation of the pre-existing $CD103^{+}$ T_{RM} cells in the inflammatory tissue condition. $CD103^{+}$ cells were observed mostly within or adjacent to the apoptotic basal cell layers as well as often in the sub-interface areas of OLP. Their locations that involved this lesional degenerated areas suggested their possible implication in the OLP pathogenesis. Moreover, $CD103^{+}$ cells in OLP in this study were barely detected in areas close to blood vessels. This phenomenon likely suggested the long-resident nature of $CD103^{+}$ cells in the peripheral tissues.

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Most studies of $CD103^+$ T_{RM} cells focused on $CD8^+CD103^+$ T_{RM} cells because the resident characteristics of T_{RM} cells was best represented in the $CD8^+$ T cell subset rather than in the $CD4^+$ T cell subset. The portions of $CD4^+CD103^+$ T_{RM} cells and $CD8^+CD103^+$ T_{RM} cells are variable in different tissues and also in different conditions[14]. Immunohistochemical analysis in this study showed superimposition of $CD103^+$ cells on both $CD4^+$ cells and $CD8^+$ cells. However, it was too crowded in the stained sections to evaluate whether $CD103^+$ cells predominantly coexpressed on $CD4^+$ cells or $CD8^+$ cells. In order to unravel this point, flow cytometry analysis of the extracted lesional T cells was additionally performed in another OLP specimen that met the inclusion criteria. The results revealed that 25.32% of lesional T cells were accounted for $CD103^+$ T_{RM} cells. $CD103^+$ T_{RM} cells represented 8.68% of $CD4^+$ cells and 37.48% of $CD8^+$ T cells, indicating $CD8^+CD103^+$ T_{RM} cells were the majority of T_{RM} cell subset in OLP [data not shown].

Notably, T_{RM} cells almost always express CD69 and often co-express CD103 as the molecular markers. CD103 is more specific to T_{RM} cells, but not all T_{RM} cells express CD103[14, 73]. Moreover, CD103 is also expressed on dendritic cells[142], mast cells[143] and macrophage[144]. However, since T cells are the most frequent cell type found in the OLP lesion[8]; thus, most $CD103^+$ cells observed in the OLP tissues are considered to be $CD103^+$ T_{RM} cells. In this study, most of $CD103^+$ cells in each OLP section exhibited quite homogeneous in shape, size and staining intensity that might imply the same type of these cells. On the contrary, $CD103^+$ cells in the normal mucosa were rather varied in shapes, sizes and staining patterns. A combination of CD103-expressing cell types were considered; however, it was difficult to clearly distinguish these cell types in the hematoxylin-stained sections without further immunologic markers.

Owing to the long-term establishment of T_{RM} cells in the peripheral tissue, they act as the first-line defense rapidly controlling local pathogen invasion by direct target cell killing together with sensing and alarm function that subsequently trigger a cascade of innate and adaptive immune functions leading to a tissue-wide state of alert and protection[88, 145]. The sensing and alarm function is distinctly effective over the direct target cell killing for the pathogen protection because this function depends on T_{RM}

cell-derived cytokines including IFN-γ, IL-2 and TNF, and does not require direct contact between T_{RM} cells and the responding cells. Therefore, even low densities of T_{RM} cells are sufficient to induce effective immune responses upon recognition of relatively few pathogens[145, 146]. However, T_{RM} cells offer not only the protective roles, but also the deleterious parts that result in the autoimmune disease[14] including OLP[2, 17].

According to this study's findings, the several-fold increase in $CD103^+$ cells that were concordant with the accumulations of $CD3^+$, $CD4^+$ and $CD8^+$ cells in OLP, together with the apoptosis-related location of $CD103^+$ cells that also corresponded to the position of $CD3^+$, $CD4^+$ and $CD8^+$ cells, suggested that $CD103^+$ cells might have an association with the OLP lesions by which they might cooperate with other T cells in driving an inflammatory process and executing an apoptotic mechanism. Although the proportion of $CD103^+$ cells were not as outstandingly high as the numbers of other T cells in OLP, based on the sensing and alarm function, this available amount of $CD103^+$ cells might be large enough to trigger the pathomechanism of OLP.

The assumption of the participation of $CD103^+$ T_{RM} cells in the OLP pathogenesis might be also supported by the evidence that the effective cytokines derived from T_{RM} cells are as same as the cytokines that are chiefly responsible for the disease mechanism of OLP. These cytokines consist of IFN-γ, IL-2 and TNF-α, and extend to a protease enzyme named granzyme B[8, 14]. In OLP, $CD103^+$ T_{RM} cells, particularly $CD8^+$ subset, may act like Th1 cells by releasing IFN-γ and IL-2 that may promote recruitment and help activation of $CD8^+$ T cells. These T_{RM} cells, acting like $CD8^+$ T cells, may also secrete TNF-α and granzyme B that may directly lyse the epithelial cells resulting in the degeneration of the basal cell layers. TNF-α combining with IFN-γ and IL-2 may additionally recruit and induce several inflammatory cells that may lead to amplification of the OLP inflammation. However, these comments require further investigation to confirm whether the mentioned cytokines and enzyme are really secreted by T_{RM} cells or constitutively released from other infiltrating $CD4^+$ and $CD8^+$ T cells.

T_{RM} cells require activation signals from TCR and probably from CD103 to release a number of cytokines and kill infected target cells[134, 147]. In addition, shortly after activation, T_{RM} cells rapidly secrete IFN-γ to broaden their protective spectrum that make them be able to provide cross-protection against antigenically unrelated

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pathogens[147]. In OLP, the pre-existing T_{RM} cells specific for a previously encountered virus may cross-react with other antigens, such as heterologous viral particles, bacterial products, contact allergens, drugs and self-antigens, in the absence of the cognate antigen, and then cause damage to the epithelial cells[2]. Viruses, such as HPV, EBV, HHV-6 and HCV, have been proposed to be a primary antigen because of their prevalence in the normal oral cavity and in OLP[2-4, 34] as well as their molecular mimicry with self-peptides[148]. Remarkably, although large parts in the oral cavity are ongoing microbial attack, T_{RM} cells can provide effective protection and control localized tissue responses in a clinical silent manner without overt symptoms[145].

Accumulating data suggest that $CD103^+$ T_{RM} cell-mediated mechanism may serve as one of the pathway partly playing a role in the OLP pathogenesis. $CD103^+$ T_{RM} cells in OLP may be malfunction and omit their protective responsibilities. They, with cross-reactivity, would rather destroy self-epithelial cells than eliminate invading pathogens. Their sensing and alarm function, on the flip side, may help initiate, render and maintain the inflammatory process in OLP.

Here, this study would like to present the hypothetic model for $CD103^+$ T_{RM} cell-mediated OLP, as follows. In the previously infected or inflamed oral mucosa, cross-reactive $CD103^+$ T_{RM} cells that recognize self-antigens may directly trigger the apoptosis of the basal epithelial cells via 3 possible mechanisms comprising TNF-α/TNFR1, granzyme B with perforin and Fas/FasL. Dead or dying epithelial cells release self-RNAs and self-DNAs that may be combined with some peptides, suspected LL-37 (leucine leucine-37 or cathelicidin antimicrobial peptide-18), and in turn activate $CD103^+$ T_{RM} cells in a vicious cycle manner. $CD103^+$ T_{RM} cells may also secrete IFN-γ combining with IL-2 and TNF-α to recruit both $CD4^+$ and $CD8^+$ circulating memory T cells to the inflamed area. $CD8^+$ memory T cells with assistance from $CD4^+$ memory T cells are stimulated to elicit further apoptosis of the basal epithelial cells. After the inflammation is suppressed, the minority of these circulating memory T cells may differentiate into secondary T_{RM} cells for further challenges *[Figure 1]*.

Note that, the scope of this study covered only the distribution and the quantity of $CD103^+$ T_{RM} cells. To prove the stated hypothesis, further investigations are required

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to demonstrate coordination between CD103$^+$ T_{RM} cells and their related substances, comprising IFN-γ, IL-2, TNF-α and granzyme B, with other factors expectedly participating in OLP, such as LL-37, self-nucleic acid, plasmacytoid dendritic cells, and especially with CD4$^+$ T cells and CD8$^+$ T cells which are the principal cells in the OLP disease mechanism. An integration of experimental and clinical studies will be useful to examine clinical potential of CD103$^+$ T_{RM} cells for occurrence of OLP as well as to explore possibility of harnessing CD103$^+$ T_{RM} cell biology for therapeutic application.

Some difficulties in carrying out this study were found in the immunohistochemical staining process which include selection of a potential anti-CD103 primary antibody, detailed modification of an immunohistochemical technique for optimal staining, and prevention of section detachment after antigen retrieval. Manual counting of all the positive cells, which are numerous and dense like sand on the seashore, in the OLP tissues particularly within the lymphocytic bands is another challenge in this study. Moreover, a flood of articles on T_{RM} cells that used various approach methods, conducted in different kinds of animals, focused on different tissues and discussed heterogenous subsets of T_{RM} cells made them complicated to understand and review. Despite that, a very small number of the studies on T_{RM} cells in the OLP lesions were published.

Even though there are a few studies raising a question whether CD103$^+$ T_{RM} cells play more important protective roles over the circulating memory T cell population in the peripheral tissues. More recent review articles have confirmed the long-resident habit, the effective defensive functions as well as the therapeutic deployment of CD103$^+$ T_{RM} cells[134, 145, 149-151].

Presently, the wide appreciation that CD103$^+$ T_{RM} cells are a crucial component of the peripheral tissue immunity has pushed them as a promising strategic target for vaccines and immunotherapies against infection, cancer and chronic inflammation[145]. This current study is the first report in the Thai patients providing primary information that CD103$^+$ T_{RM} cells may be associated with the pathogenesis of OLP. However, much remains to be explored regarding their roles in the disease mechanism. The growing knowledge about their roles in OLP will help fulfil a current concept of the OLP pathogenesis and put forward to the therapeutic and prophylactic strategies for OLP.

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CHAPTER VI

CONCLUSION

Since the OLP lesions demonstrate the several-fold increase in the density of CD103$^+$ cells together with the locations of these cells that mostly involve the lesional degenerated area, the current study suggests that CD103$^+$ T_{RM} cells may be associated with the pathogenesis of OLP.

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APPENDIX

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Appendix 1 Characteristics data of OLP patients

Case	Age (years)	Sex	Chief complaint	Duration (months)	Type	Affected sites	Biopsy site (Buccal mucosa)
1	60	Female	Ulceration	7	Ulcerative	Buccal mucosa, gingiva, tongue	Left
2	48	Female	Burning sensation	12	Atrophic	Buccal mucosa, gingiva, mucobuccal fold, floor of mouth	Right
3	45	Female	Burning sensation	6	Reticular	Buccal mucosa, gingiva, tongue	Left
4	50	Female	Detected by dentist	Not known	Atrophic	Buccal mucosa, gingiva, mucobuccal fold	Left
5	78	Female	Burning sensation	2	Ulcerative	Buccal mucosa, gingiva, mucobuccal fold, hard palate	Right
6	68	Female	Burning sensation	6	Atrophic	Buccal mucosa, gingiva, tongue	Right
7	45	Female	Ulceration	8	Ulcerative	Buccal mucosa, gingiva, tongue, lip	Left
8	46	Female	Burning sensation	6	Ulcerative	Buccal mucosa, gingiva, mucobuccal fold, tongue	Left
9	41	Female	Ulceration	0.5	Ulcerative	Buccal mucosa, tongue	Right
10	62	Female	Detected by dentist	Not known	Atrophic	Buccal mucosa, gingiva, mucobuccal fold, tongue	Left
11	67	Female	Burning sensation	12	Atrophic	Buccal mucosa, mucobuccal fold	Right
12	54	Male	Roughness	1	Ulcerative	Buccal mucosa	Right
13	36	Male	Ulceration	0.17	Reticular	Buccal mucosa, mucobuccal fold, tongue, hard palate, lip	Right
14	59	Female	Burning sensation	12	Ulcerative	Buccal mucosa, gingiva, mucobuccal fold	Right
15	55	Female	Ulceration	12	Atrophic	Buccal mucosa, gingiva	Left

Appendix 2 Characteristics data of normal subjects

Case	Age (years)	Sex	Biopsy site (Area of mucosa in buccal region)
1	20	Female	38
2	39	Female	48
3	23	Female	48
4	28	Male	48
5	21	Female	48
6	20	Female	48
7	17	Female	48
8	24	Female	48
9	21	Female	48
10	23	Female	38
11	23	Female	48
12	22	Male	48
13	20	Female	48
14	21	Female	48
15	19	Female	48

Appendix 3 Areas (mm^2) and numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells (cells) in OLP samples

Case	Area (mm^2)			Number of positive cells (cells)											
	Epithelium	Lamina propria	Epithelium + lamina propria	Epithelium				Lamina propria				Epithelium + lamina propria			
				CD3	CD4	CD8	CD103	CD3	CD4	CD8	CD103	CD3	CD4	CD8	CD103
1	0.86	0.31	1.17	286	107	129	56	1763	664	921	173	2049	771	1050	229
2	1.68	0.8	2.48	276	118	137	89	6102	2173	3550	589	6378	2291	3687	678
3	1.67	0.25	1.92	208	79	115	75	2120	783	1164	223	2328	862	1279	298
4	1.25	0.39	1.64	441	114	187	184	3241	1288	1765	416	3682	1402	1952	600
5	0.49	0.35	0.84	32	12	15	17	2925	1270	1584	296	2957	1282	1599	313
6	0.91	0.50	1.41	442	226	192	161	3726	1883	1674	510	4168	2109	1866	671
7	0.91	1.03	1.94	313	103	198	117	8122	3498	4038	562	8435	3601	4236	679
8	1.24	0.61	1.85	133	40	76	47	5575	2523	2962	1129	5708	2563	3038	1176
9	0.33	0.15	0.48	335	110	136	52	1304	548	729	74	1639	658	865	126
10	0.49	0.33	0.82	94	24	50	49	1989	1123	841	485	2083	1147	891	534
11	0.56	0.43	0.99	200	52	108	54	4032	902	2568	714	4232	954	2676	768
12	0.68	0.80	1.48	128	60	64	56	5120	1573	2985	538	5248	1633	3049	594
13	0.65	0.47	1.12	231	110	114	54	3653	1622	1932	416	3884	1732	2046	470
14	0.29	0.40	0.69	127	24	54	43	2514	917	1566	310	2641	941	1620	353
15	1.03	0.43	1.46	144	52	68	48	2874	990	1368	226	3018	1042	1436	274

Appendix 4 Areas (mm^2) and numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells (cells) in normal mucosa samples

Case	Area (mm^2)			Number of positive cells (cells)											
	Epithelium	Lamina propria	Epithelium + lamina propria	Epithelium				Lamina propria				Epithelium + lamina propria			
				CD3	CD4	CD8	CD103	CD3	CD4	CD8	CD103	CD3	CD4	CD8	CD103
1	0.94	0.62	1.56	89	47	31	16	63	34	21	26	152	81	52	42
2	1.62	1.61	3.23	73	32	18	21	142	72	47	46	215	104	65	67
3	0.69	0.56	1.25	18	8	7	6	28	14	5	7	46	22	12	13
4	1.75	1.31	3.06	5	2	2	1	37	18	12	6	42	20	14	7
5	1.75	1.82	3.57	60	31	25	17	116	66	38	72	176	97	63	89
6	0.72	0.62	1.34	32	14	10	6	51	26	12	17	83	40	22	23
7	1.40	1.67	3.07	61	31	25	14	83	44	31	28	144	75	56	42
8	0.50	0.54	1.04	30	14	8	8	45	21	14	17	75	35	22	25
9	1.61	1.20	2.81	72	39	28	34	114	73	25	52	186	112	53	86
10	1.23	1.19	2.42	89	39	24	8	137	82	34	17	226	121	58	25
11	0.56	1.47	2.03	14	7	4	3	42	21	17	10	56	28	21	13
12	1.92	1.81	3.73	64	32	23	21	121	63	55	32	185	95	78	53
13	1.22	0.73	1.95	51	36	10	18	92	57	29	17	143	93	39	35
14	1.27	1.36	2.63	16	10	4	6	34	18	9	8	50	28	13	14
15	0.83	0.54	1.37	12	7	3	4	52	34	14	17	64	41	17	21

Appendix 5 Descriptive statistics: areas (mm^2) and numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells (cells) in OLP and normal mucosa samples

Part	Area and positive cells	OLP				Normal mucosa			
		N	Mean	Std. deviation	Std. error mean	N	Mean	Std. deviation	Std. error mean
Epithelium	Area	15	.87	.439	.113	15	1.20	0.471	0.122
	CD3	15	226.00	122.268	31.570	15	45.73	29.117	7.518
	CD4	15	82.07	54.363	14.037	15	23.27	14.772	3.814
	CD8	15	109.53	55.422	14.310	15	14.80	10.339	2.669
	CD103	15	73.47	46.223	11.935	15	12.20	8.994	2.322
Lamina propria	Area	15	.48	.235	.061	15	1.14	0.491	0.127
	CD3	15	3670.67	1866.486	481.925	15	77.13	40.096	10.353
	CD4	15	1450.47	799.036	206.310	15	42.87	23.751	6.133
	CD8	15	1976.47	1018.218	262.903	15	24.20	14.620	3.775
	CD103	15	444.07	259.131	66.907	15	24.80	18.816	4.858
Epithelium + lamina propria	Area	15	1.35	.552	.143	15	2.34	0.897	0.232
	CD3	15	3896.67	1881.974	485.924	15	122.87	66.046	17.053
	CD4	15	1532.53	812.664	209.829	15	66.13	36.473	9.417
	CD8	15	2086.00	1033.568	266.866	15	39.00	22.684	5.857
	CD103	15	517.53	267.237	69.000	15	37.00	26.235	6.774

Appendix 6 Numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cells/mm^2) in OLP samples

Case	Epithelium (cells/mm^2)				Lamina propria (cells/mm^2)				Epithelium + lamina propria (cells/mm^2)			
	CD3	**CD4**	**CD8**	**C103**	**CD3**	**CD4**	**CD8**	**C103**	**CD3**	**CD4**	**CD8**	**C103**
1	332.56	124.42	150.00	65.12	5687.10	2141.94	2970.97	558.06	1751.28	658.97	897.44	195.73
2	164.29	70.24	81.55	52.98	7627.50	2716.25	4437.50	736.25	2571.77	923.79	1486.69	273.39
3	124.55	47.31	68.86	44.91	8480.00	3132.00	4656.00	892.00	1212.50	448.96	666.15	155.21
4	352.80	91.20	149.60	147.20	8310.26	3302.56	4525.64	1066.67	2245.12	854.88	1190.24	365.85
5	65.31	24.49	30.61	34.69	8357.14	3628.57	4525.71	845.71	3520.24	1526.19	1903.57	372.62
6	485.71	248.35	210.99	176.92	7452.00	3766.00	3348.00	1020.00	2956.03	1495.74	1323.40	475.89
7	343.96	113.19	217.58	128.57	7885.44	3396.12	3920.39	545.63	4347.94	1856.19	2183.51	350.00
8	107.26	32.26	61.29	37.90	9139.34	4136.07	4855.74	1850.82	3085.41	1385.41	1642.16	635.68
9	1015.15	333.33	412.12	157.58	8693.33	3653.33	4860.00	493.33	3414.58	1370.83	1802.08	262.50
10	191.84	48.98	102.04	100.00	6027.27	3403.03	2548.48	1469.70	2540.24	1398.78	1086.59	651.22
11	357.14	92.86	192.86	96.43	9376.74	2097.67	5972.09	1660.47	4274.75	963.64	2703.03	775.76
12	188.24	88.24	94.12	82.35	6400.00	1966.25	3731.25	672.50	3545.95	1103.38	2060.14	401.35
13	355.38	169.23	175.38	83.08	7772.34	3451.06	4110.64	885.11	3467.86	1546.43	1826.79	419.64
14	437.93	82.76	186.21	148.28	6285.00	2292.50	3915.00	775.00	3827.54	1363.77	2347.83	511.59
15	139.81	50.49	66.02	46.60	6683.72	2302.33	3181.40	525.58	2067.12	713.70	983.56	187.67

Appendix 7 Numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cells/mm^2) in normal mucosa samples

Case	Epithelium (cells/mm^2)				Lamina propria (cells/mm^2)				Epithelium + lamina propria (cells/mm^2)			
	CD3	CD4	CD8	C103	CD3	CD4	CD8	C103	CD3	CD4	CD8	C103
1	94.68	50.00	32.98	17.02	101.61	54.84	33.87	41.94	97.44	51.92	33.33	26.92
2	45.06	19.75	11.11	12.96	88.20	44.72	29.19	28.57	66.56	32.20	20.12	20.74
3	26.09	11.59	10.14	8.70	50.00	25.00	8.93	12.50	36.80	17.60	9.60	10.40
4	2.86	1.14	1.14	0.57	28.24	13.74	9.16	4.58	13.73	6.54	4.58	2.29
5	34.29	17.71	14.29	9.71	63.74	36.26	20.88	39.56	49.30	27.17	17.65	24.93
6	44.44	19.44	13.89	8.33	82.26	41.94	19.35	27.42	61.94	29.85	16.42	17.16
7	43.57	22.14	17.86	10.00	49.70	26.35	18.56	16.77	46.91	24.43	18.24	13.68
8	60.00	28.00	16.00	16.00	83.33	38.89	25.93	31.48	72.12	33.65	21.15	24.04
9	44.72	24.22	17.39	21.12	95.00	60.83	20.83	43.33	66.19	39.86	18.86	30.60
10	72.36	31.71	19.51	6.50	115.13	68.91	28.57	14.29	93.39	50.00	23.97	10.33
11	25.00	12.50	7.14	5.36	28.57	14.29	11.56	6.80	27.59	13.79	10.34	6.40
12	33.33	16.67	11.98	10.94	66.85	34.81	30.39	17.68	49.60	25.47	20.91	14.21
13	41.80	29.51	8.20	14.75	126.03	78.08	39.73	23.29	73.33	47.69	20.00	17.95
14	12.60	7.87	3.15	4.72	25.00	13.24	6.62	5.88	19.01	10.65	4.94	5.32
15	14.46	8.43	3.61	4.82	96.30	62.96	25.93	31.48	46.72	29.93	12.41	15.33

Appendix 8 Descriptive statistics: numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cells/mm^2) in OLP and normal mucosa samples

Part	Positive cells	OLP				Normal mucosa			
		N	Mean	Std. deviation	Std. error mean	N	Mean	Std. deviation	Std. error mean
Epithelium	CD3	15	310.80	234.002	60.419	15	39.68	23.600	6.093
	CD4	15	107.82	84.796	21.894	15	20.05	11.935	3.082
	CD8	15	146.62	95.050	24.542	15	12.56	7.969	2.058
	CD103	15	93.51	47.825	12.348	15	10.10	5.497	1.419
Lamina propria	CD3	15	7611.81	1160.267	299.580	15	73.33	31.995	8.261
	CD4	15	3025.71	707.517	182.680	15	40.99	20.700	5.345
	CD8	15	4103.92	875.561	226.069	15	21.97	9.835	2.539
	CD103	15	933.12	420.727	108.631	15	23.04	13.062	3.373
Epithelium + lamina propria	CD3	15	2988.56	913.503	235.865	15	54.71	24.621	6.357
	CD4	15	1174.04	398.041	102.774	15	29.38	13.898	3.588
	CD8	15	1606.88	584.327	150.873	15	16.83	7.520	1.942
	CD103	15	402.27	182.142	47.029	15	16.02	8.329	2.150

Appendix 9 Test of normality (Shapiro-Wilk): numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cells/mm^2) in OLP and normal mucosa samples

Part	Positive cells	OLP			Normal mucosa		
		Statistic	df	Sig.	Statistic	df	Sig.
Epithelium	CD3	.794	15	.003*	.950	15	.527
	CD4	.812	15	.005*	.951	15	.534
	CD8	.866	15	.030*	.935	15	.321
	CD103	.918	15	.177	.980	15	.971
Lamina propria	CD3	.951	15	.542	.950	15	.527
	CD4	.909	15	.132	.950	15	.519
	CD8	.975	15	.923	.960	15	.697
	CD103	.867	15	.031*	.941	15	.391
Epithelium + lamina propria	CD3	.969	15	.838	.969	15	.843
	CD4	.953	15	.579	.961	15	.709
	CD8	.981	15	.975	.947	15	.474
	CD103	.950	15	.531	.978	15	.956

Appendix 10 Unpaired t-test: differences of numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cells/mm^2) between OLP and normal mucosa samples

Part	Positive cells		Levene's test for equality of variances		t-test for equality of means						
			F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference: Lower	95% Confidence interval of the difference: Upper
Epithelium	CD103	Equal variances assumed	33.166	.000	6.710	28	.000	83.407	12.430	57.946	108.868
		Equal variances not assumed			6.710	14.370	.000*	83.407	12.430	56.813	110.002
Lamina propria	CD3	Equal variances assumed	34.053	.000	25.154	28	.000	7538.481	299.694	6924.587	8152.376
		Equal variances not assumed			25.154	14.021	.000*	7538.481	299.694	6895.794	8181.169
	CD4	Equal variances assumed	59.596	.000	16.332	28	.000	2984.721	182.758	2610.358	3359.085
		Equal variances not assumed			16.332	14.024	.000*	2984.721	182.758	2592.807	3376.636
	CD8	Equal variances assumed	25.314	.000	18.055	28	.000	4081.954	226.083	3618.844	4545.064
		Equal variances not assumed			18.055	14.004	.000*	4081.954	226.083	3597.065	4566.843

Part	Positive cells		Levene's test for equality of variances		t-test for equality of means						
					t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference	
			F	Sig.						Lower	Upper
Epithelium + lamina propria	CD3	Equal variances assumed	32.399	.000	12.434	28	.000	2933.847	235.951	2450.523	3417.171
		Equal variances not assumed			12.434	14.020	.000*	2933.847	235.951	2427.851	3439.842
	CD4	Equal variances assumed	47.119	.000	11.131	28	.000	1144.661	102.836	934.010	1355.311
		Equal variances not assumed			11.131	14.034	.000*	1144.661	102.836	924.149	1365.172
	CD8	Equal variances assumed	36.703	.000	10.538	28	.000	1590.044	150.885	1280.970	1899.118
		Equal variances not assumed			10.538	14.005	.000*	1590.044	150.885	1266.438	1913.650
	CD103	Equal variances assumed	22.580	.000	8.205	28	.000	386.253	47.078	289.819	482.688
		Equal variances not assumed			8.205	14.059	.000*	386.253	47.078	285.321	487.186

Appendix 11 Mann-Whitney U-test: differences of numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area ($cells/mm^2$) between OLP and normal mucosa samples

Ranks

Part	Positive cells	Group	N	Mean rank	Sum of ranks
Epithelium	CD3	OLP	15	22.87	343.00
		Normal	15	8.13	122.00
		Total	30		
	CD4	OLP	15	22.53	338.00
		Normal	15	8.47	127.00
		Total	30		
	CD8	OLP	15	22.93	344.00
		Normal	15	8.07	121.00
		Total	30		
Lamina propria	CD103	OLP	15	23.00	345.00
		Normal	15	8.00	120.00
		Total	30		

Test statistics[a]

	Epithelium			Lamina propria
	CD3	CD4	CD8	CD103
Mann-Whitney U	2.000	7.000	1.000	0.000
Wilcoxon W	122.000	127.000	121.000	120.000
Z	-4.583	-4.376	-4.625	-4.667
Asymp. Sig. (2-tailed)	.000*	.000*	.000*	.000*
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]	.000[b]	.000[b]

a. Grouping Variable: Group
b. Not corrected for ties

Appendix 12 Wilcoxon Signed Ranks test: differences of numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cells/mm^2) between epithelium and lamina propria in OLP samples

Ranks

Positive cells			N	Mean rank	Sum of ranks
Epithelium	Lamina propria				
CD3	CD3	Negative Ranks	0[a]	0.00	0.00
		Positive Ranks	15[b]	8.00	120.00
		Ties	0[c]		
		Total	15		
CD4	CD4	Negative Ranks	0[d]	0.00	0.00
		Positive Ranks	15[e]	8.00	120.00
		Ties	0[f]		
		Total	15		
CD8	CD8	Negative Ranks	0[g]	0.00	0.00
		Positive Ranks	15[h]	8.00	120.00
		Ties	0[i]		
		Total	15		
CD103	CD103	Negative Ranks	0[j]	0.00	0.00
		Positive Ranks	15[k]	8.00	120.00
		Ties	0[l]		
		Total	15		

a. Lamina propria, CD3 < Epithelium, CD3
b. Lamina propria, CD3 > Epithelium, CD3
c. Lamina propria, CD3 = Epithelium, CD3
d. Lamina propria, CD4 < Epithelium, CD4
e. Lamina propria, CD4 > Epithelium, CD4
f. Lamina propria, CD4 = Epithelium, CD4
g. Lamina propria, CD8 < Epithelium, CD8
h. Lamina propria, CD8 > Epithelium, CD8
i. Lamina propria, CD8 = Epithelium, CD8
j. Lamina propria, CD103 < Epithelium, CD103
k. Lamina propria, CD103 > Epithelium, CD103
l. Lamina propria, CD103 = Epithelium, CD103

Test statistics[a]

Positive cells		Z	Asymp. Sig. (2-tailed)
Epithelium	Lamina propria		
CD3	CD3	-3.408[b]	.001*
CD4	CD4	-3.408[b]	.001*
CD8	CD8	-3.408[b]	.001*
CD103	CD103	-3.408[b]	.001*

a. Wilcoxon Signed Ranks Test
b. Based on negative ranks

Appendix 13 Paired t-test: differences of numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD103^+$ cells per area (cells/mm^2) between epithelium and lamina propria in normal mucosa samples

Paired samples correlations

Pair	Positive cells		N	Correlation	Sig.
	Epithelium	Lamina propria			
Pair 1	CD3	CD3	15	.646	.009
Pair 2	CD4	CD4	15	.605	.017
Pair 3	CD8	CD8	15	.478	.072
Pair 4	CD103	CD103	15	.746	.001

Paired samples test

Pair	Positive cells		Paired differences					t	df	Sig. (2-tailed)
	Epithelium	Lamina propria	Mean	Std. deviation	Std. error mean	95% Confidence interval of the difference				
						Lower	Upper			
Pair 1	CD3	CD3	-33.647	24.586	6.348	-47.262	-20.031	-5.300	14	.000*
Pair 2	CD4	CD4	-20.945	16.495	4.259	-30.080	-11.810	-4.918	14	.000*
Pair 3	CD8	CD8	-9.407	9.239	2.386	-14.524	-4.291	-3.943	14	.001*
Pair 4	CD103	CD103	-12.938	9.680	2.499	-18.298	-7.578	-5.177	14	.000*

Appendix 14 Proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) in OLP samples

Case	Epithelium (%)			Lamina propria (%)			Epithelium + lamina propria (%)		
	CD4/CD3	CD8/CD3	CD103/CD3	CD4/CD3	CD8/CD3	CD103/CD3	CD4/CD3	CD8/CD3	CD103/CD3
1	37.41	45.10	19.58	37.66	52.24	9.81	37.63	51.24	11.18
2	42.75	49.64	32.25	35.61	58.18	9.65	35.92	57.81	10.63
3	37.98	55.29	36.06	36.93	54.91	10.52	37.03	54.94	12.80
4	25.85	42.40	41.72	39.74	54.46	12.84	38.08	53.01	16.30
5	37.50	46.88	53.13	43.42	54.15	10.12	43.35	54.08	10.59
6	51.13	43.44	36.43	50.54	44.93	13.69	50.60	44.77	16.10
7	32.91	63.26	37.38	43.07	49.72	6.92	42.69	50.22	8.05
8	30.08	57.14	35.34	45.26	53.13	20.25	44.90	53.22	20.60
9	32.84	40.60	15.52	42.02	55.90	5.67	40.15	52.78	7.69
10	25.53	53.19	52.13	56.46	42.28	24.38	55.06	42.77	25.64
11	26.00	54.00	27.00	22.37	63.69	17.71	22.54	63.23	18.15
12	46.88	50.00	43.75	30.72	58.30	10.51	31.12	58.10	11.32
13	47.62	49.35	23.38	44.40	52.89	11.39	44.59	52.68	12.10
14	18.90	42.52	33.86	36.48	62.29	12.33	35.63	61.34	13.37
15	36.11	47.22	33.33	34.45	47.60	7.86	34.53	47.58	9.08

Appendix 15 Proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) in normal mucosa samples

Case	Epithelium (%)			Lamina propria (%)			Epithelium + lamina propria (%)		
	CD4/CD3	CD8/CD3	CD103/CD3	CD4/CD3	CD8/CD3	CD103/CD3	CD4/CD3	CD8/CD3	CD103/CD3
1	52.81	34.83	17.98	53.97	33.33	41.27	53.29	34.21	27.63
2	43.84	24.66	28.77	50.70	33.10	32.39	48.37	30.23	31.16
3	44.44	38.89	33.33	50.00	17.86	25.00	47.83	26.09	28.26
4	40.00	40.00	20.00	48.65	32.43	16.22	47.62	33.33	16.67
5	51.67	41.67	28.33	56.90	32.76	62.07	55.11	35.80	50.57
6	43.75	31.25	18.75	50.98	23.53	33.33	48.19	26.51	27.71
7	50.82	40.98	22.95	53.01	37.35	33.73	52.08	38.89	29.17
8	46.67	26.67	26.67	46.67	31.11	37.78	46.67	29.33	33.33
9	54.17	38.89	47.22	64.04	21.93	45.61	60.22	28.49	46.24
10	43.82	26.97	8.99	59.85	24.82	12.41	53.54	25.66	11.06
11	50.00	28.57	21.43	50.00	40.48	23.81	50.00	37.50	23.21
12	50.00	35.94	32.81	52.07	45.45	26.45	51.35	42.16	28.65
13	70.59	19.61	35.29	61.96	31.52	18.48	65.03	27.27	24.48
14	62.50	25.00	37.50	52.94	26.47	23.53	56.00	26.00	28.00
15	58.33	25.00	33.33	65.38	26.92	32.69	64.06	26.56	32.81

Appendix 16 Descriptive statistics: proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) in OLP and normal mucosa samples

Part	Positive cells	OLP				Normal mucosa			
		N	Mean	Std. deviation	Std. error mean	N	Mean	Std. deviation	Std. error mean
Epithelium	CD4/CD3	15	35.30	9.213	2.379	15	50.89	8.122	2.097
	CD8/CD3	15	49.34	6.333	1.635	15	31.93	7.211	1.862
	CD103/CD3	15	34.72	10.622	2.743	15	27.56	9.533	2.461
Lamina propria	CD4/CD3	15	39.94	8.144	2.103	15	54.47	5.813	1.501
	CD8/CD3	15	53.65	5.888	1.520	15	30.60	7.260	1.875
	CD103/CD3	15	12.24	5.059	1.306	15	30.98	12.629	3.261
Epithelium + lamina propria	CD4/CD3	15	39.59	7.873	2.033	15	53.29	5.883	1.519
	CD8/CD3	15	53.18	5.610	1.448	15	31.20	5.400	1.394
	CD103/CD3	15	13.57	4.973	1.284	15	29.26	9.756	2.519

Appendix 17 Test of normality (Shapiro-Wilk): proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) in OLP and normal mucosa samples

Part	Positive cells	OLP			Normal mucosa		
		Statistic	df	Sig.	Statistic	df	Sig.
Epithelium	CD3	.971	15	.871	.918	15	.181
	CD4	.958	15	.649	.916	15	.168
	CD8	.965	15	.777	.982	15	.980
	CD103	.978	15	.950	.906	15	.116
Lamina propria	CD3	.975	15	.926	.977	15	.948
	CD4	.894	15	.078	.948	15	.488
	CD8	.974	15	.914	.889	15	.065
	CD103	.973	15	.905	.884	15	.054
Epithelium + lamina propria	CD3	.908	15	.124	.912	15	.143
	CD4	.971	15	.871	.918	15	.181
	CD8	.958	15	.649	.916	15	.168
	CD103	.965	15	.777	.982	15	.980

Appendix 18 Unpaired t-test: differences of proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) between OLP and normal mucosa samples

Part	Positive cells		Levene's test for equality of variances		t-test for equality of means						
					t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference	
			F	Sig.						Lower	Upper
Epithelium	CD4/ CD3	Equal variances assumed	.511	.481	-4.918	28	.000*	-15.595	3.171	-22.091	-9.099
		Equal variances not assumed			-4.918	27.567	.000	-15.595	3.171	-22.095	-9.094
	CD8/ CD3	Equal variances assumed	1.314	.261	7.025	28	.000*	17.407	2.478	12.331	22.483
		Equal variances not assumed			7.025	27.541	.000	17.407	2.478	12.327	22.486
	CD103/ CD3	Equal variances assumed	.014	.908	1.945	28	.062	7.167	3.685	-0.381	14.716
		Equal variances not assumed			1.945	27.679	.062	7.167	3.685	-0.385	14.720

Part	Positive cells		Levene's test for equality of variances		t-test for equality of means						
			F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference: Lower	95% Confidence interval of the difference: Upper
Lamina propria	CD4/ CD3	Equal variances assumed	.710	.407	-5.625	28	.000*	-14.533	2.584	-19.825	-9.241
		Equal variances not assumed			-5.625	25.326	.000	-14.533	2.584	-19.850	-9.215
	CD8/ CD3	Equal variances assumed	.705	.408	9.546	28	.000*	23.041	2.414	18.097	27.985
		Equal variances not assumed			9.546	26.855	.000	23.041	2.414	18.087	27.994
	CD103/ CD3	Equal variances assumed	6.702	.015	-5.335	28	.000	-18.741	3.513	-25.937	-11.546
		Equal variances not assumed			-5.335	18.381	.000*	-18.741	3.513	-26.110	-11.372

Part	Positive cells		Levene's test for equality of variances		t-test for equality of means						
					t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference	
			F	Sig.						Lower	Upper
Epithelium + lamina propria	CD4/ CD3	Equal variances assumed	.721	.403	-5.400	28	.000*	-13.703	2.538	-18.901	-8.505
		Equal variances not assumed			-5.400	25.920	.000	-13.703	2.538	-18.920	-8.486
	CD8/ CD3	Equal variances assumed	.240	.628	10.934	28	.000*	21.983	2.011	17.864	26.101
		Equal variances not assumed			10.934	27.960	.000	21.983	2.011	17.864	26.101
	CD103/ CD3	Equal variances assumed	1.570	.221	-5.549	28	.000*	-15.690	2.827	-21.481	-9.899
		Equal variances not assumed			-5.549	20.816	.000	-15.690	2.827	-21.573	-9.807

Appendix 19 Paired t-test: differences of proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) between epithelium and lamina propria in OLP samples

Paired samples correlations

Pair	Positive cells		N	Correlation	Sig.
	Epithelium	Lamina			
Pair 1	CD4/CD3	CD4/CD3	15	.090	.750
Pair 2	CD8/CD3	CD8/CD3	15	-.113	.688
Pair 3	CD103/CD3	CD103/CD3	15	.392	.149

Paired samples test

Pair	Positive cells		Paired differences					t	df	Sig. (2-tailed)
	Epithelium	Lamina propria	Mean	Std. deviation	Std. error mean	95% Confidence interval of the difference				
						Lower	Upper			
Pair 1	CD4/CD3	CD4/CD3	-4.643	11.736	3.030	-11.142	1.856	-1.532	14	.148
Pair 2	CD8/CD3	CD8/CD3	-4.309	9.122	2.355	-9.361	0.742	-1.830	14	.089
Pair 3	CD103/CD3	CD103/CD3	22.481	9.815	2.534	17.045	27.916	8.871	14	.000*

Appendix 20 Paired t-test: differences of proportions of $CD4^+$, $CD8^+$ and $CD103^+$ cells to $CD3^+$ cells (%) between epithelium and lamina propria in normal mucosa samples

Paired samples correlations

Pair	Positive cells		N	Correlation	Sig.
	Epithelium	Lamina			
Pair 1	CD4/CD3	CD4/CD3	15	.587	.021
Pair 2	CD8/CD3	CD8/CD3	15	.051	.856
Pair 3	CD103/CD3	CD103/CD3	15	.271	.329

Paired samples test

Pair	Positive cells		Paired differences					t	df	Sig. (2-tailed)
	Epithelium	Lamina propria	Mean	Std. deviation	Std. error mean	95% Confidence interval of the difference				
						Lower	Upper			
Pair 1	CD4/CD3	CD4/CD3	-3.581	6.657	1.719	-7.267	0.106	-2.083	14	.056
Pair 2	CD8/CD3	CD8/CD3	1.325	9.967	2.574	-4.195	6.844	.515	14	.615
Pair 3	CD103/CD3	CD103/CD3	-3.428	13.607	3.513	-10.963	4.107	-.976	14	.346

VITA

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