Qualitative and Quantitative Assessment of Immune Cells in Oral Mucosal Lichen Planus (OMLP)

(Penilaian Sel-sel Imun di dalam Mukosa Mulut *Lichen Planus* (OMLP) Secara Kualitatif dan Kuantitatif)

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ABSTRACT

Cell mediated immunity is currently thought to be involved in the pathogenesis of oral mucosal lichen planus (OMLP). However, literature reveals there is no large scale data of immunohistochemistry (IHC) study on these immune cell populations. The aim of this study was to assess and compare immune cell surface identification markers CD3, CD4, CD8, CD19 and CD83 between the OMLP (n=40) and non-specific inflammatory lesions (as control group) (n=10) qualitatively and quantitatively. Kruskal-Wallis and Mann Whitney U tests have been used to make comparison between the test and control group, p values of less than 0.05 was considered to be statistically significant. T cell surface markers (CD3+, CD4+ and CD8+), B cells (CD19+) and mature dendritic cells (CD83+) showed intense immunostaining in OMLP tissues with a significantly higher expression of positive cells than in the control group (p<0.05). CD3, CD4 and CD8+ve T cells were the predominant inflammatory cell type in OMLP rather than CD19+ B cells, supporting the role of Th1 cells in the pathogenesis of OMLP. CD83+ mature dendritic cells were present in the least number and were mostly localized to areas where there were aggregates of lymphocyte. There was a positive correlation and direct relationship between T and B lymphocyte subsets whereby as one subset increased, the other follows.

Keywords: Immune cells; immunohistochemistry (IHC); oral mucosal lichen planus (OMLP)

ABSTRAK

Imuniti selular telah diketahui umum terlibat dalam patogenesis lichen planus *(OMLP). Walau bagaimanapun, sehingga kini tiada kajian imunohistokimia yang membentangkan pangkalan data secara besar mengenai populasi sel-sel imun di dalam OMLP. Matlamat kajian ini adalah untuk menganalisis dan membandingkan identiti permukaaan sel imun CD3, CD4, CD8, CD19 dan CD83 antara OMLP (n=40) dan kumpulan dengan inflamasi tidak spesifik sebagai kawalan (n=10) secara kuantitatif dan kualitatif. Ujian statistik Kruskal-Wallis dan Mann Whitney U telah digunakan untuk membuat perbandingan antara kumpulan kajian dan kumpulan kawalan, nilai p yang kurang daripada 0.05 dianggap nilai statistik yang signifikan. Kami mendapati sel yang mempunyai identiti permukaan sel T (CD3+, CD4+ dan CD8+), sel B (CD19+) dan sel dendritik yang matang (CD83+) menunjukkan intensiti 'immunostaining' yang kuat di dalam tisu OMLP iaitu sel yang menunjukkan ekspresi positif berbanding dengan kumpulan kawalan (p<0.05). Sel T yang positif dengan identiti permukaan CD3, CD4 and CD8 adalah sel inflamasi utama di dalam OMLP berbanding dengan sel B dengan identiti permukaan CD19+. Ini menguatkan lagi teori bahawa sel Th1 amat penting dalam patogenesis OMLP. Sel dendritik matang CD83+ hadir dalam jumlah yang kecil dan kebanyakannya tertumpu di kawasan sel limfosit bergumpalan. Terdapat kolerasi positif dan hubungan terus antara subset sel-sel limfosit T dan B, jika satu subset bertambah maka subset yang lain juga turut bertambah.*

Kata kunci: Imunohistokimia (IHC); mukosa mulut lichen planus *(OMLP); sel imun*

INTRODUCTION

The exact aetiology and pathogenesis of OMLP remain unknown but it appears to be complex and multi-factorial. Accumulating evidence suggests there is an immunological component, particularly cell-mediated immunity that is directly involved in the pathogenesis of the OMLP. Many authors in the literature support the hypothesis that the innate immune response plays an essential role in triggering the cascades of exclusively Th1 cell phenotypes which result in the chronic exacerbations of OMLP (Porter et al. 1997; Walsh et al. 1990; Walton et al. 1998). In comparison, Th2 cells induce humoral immunity and there is relatively little evidence available to support their role in the pathogenesis of OMLP (Porter et al. 1997).

Antigen presenting cells (APCs) play an important role in navigating the immune response, moderating the antigen presentation and providing powerful signals for the expansion of these autoreactive T cells (Porter et al. 1997; Sugerman et al. 2000). Under the influence of Th1 cytokines, chemokines and their respective receptor,

66

this promotes the recruitment of effectors T cells and induces the keratinocyte capase cascade and causes basal keratinocytes apoptosis (Khan et al. 2003; Lodi et al. 2005).

The aim of this study was to assess and compare the immune cell surface identification markers (CD3, CD4, CD8, CD19 and CD83) between the OMLP and non-specific inflammatory control group qualitatively and quantitatively.

MATERIALS AND METHODS

SAMPLE SELECTION

For both experimental (OMLP) and control (normal or hyperplastic oral mucosa with non-specific inflammation) groups cases were selected from the archives of the MedLab Dental Oral Pathology Diagnostic Service, Faculty of Dentistry, University of Otago. All cases were reviewed to ensure that the diagnosis were conformed to the histopathological criteria as defined by the WHO Histological Typing of Cancer and Precancer of the Oral Mucosa (Pindborg et al. 1997) and that adequate clinical information was available (age, gender, past medical history and clinical site of the oral lesion). Exclusion criteria were: medically compromised patients or those currently on medications, specimens that were associated with amalgam restorations or had histological evidence of epithelial dysplasia or ulceration. The OMLP cases were sub-categorised according to epithelial thickness (atrophic or hyperplastic) as determined histologically. To allow comparison of immunohistochemistry staining, fifty formalin-fixed paraffin-embedded (FFPE) archived tissue specimens were selected and divided into three main groups: control group (n=10) which were samples of normal or hyperplastic oral mucosa with non-specific inflammation and the experimental groups: atrophic OMLP (n=20) and hyperplastic OMLP (n=20).

IMMUNOHISTOCHEMISTRY

IHC was manually performed according to a conventional protocol. FFPE tissue sections were deparaffinised, treated with heat-induced antigen retrieval technique, quenched in endogenous peroxidase enzyme for 15 min duration followed by immunostained using the following monoclonal mouse anti-human primary antibodies: Santa Cruz CD8-α/O.N.66 (1:100) DAKO CD19/LE-CD19 (1:100) and Novocastra NCL-CD83/IH4 (1:100). For both CD3/2GV6 (1:20) and CD4/SP35 (1:20) primary antibodies, pre-diluted ready to use (RTU) rabbit monoclonal antibodies, (Ventana® Medical Systems, Inc., USA) were used in an automatic immunostainer (Ventana® Benchmark XT, Roche Inc., USA), according to the manufacturer's recommendations. Positive and negative controls were used to validate the IHC run. Isotype controls of normal mouse IgG (sc-2025; Santa Cruz Biotechnology Inc., USA) and normal rabbit IgG (sc-2027; Santa Cruz Biotechnology Inc., USA) antibody were used and served as negative controls for

IHC reactions. Visualization of the antigen-antibody signal amplification was achieved by using an indirect enzyme labelled method by using a streptavidin-biotin system (Universal LSAB™2Kit/HRP, DakoCytomation, Denmark) and 3, 3'diaminobenzidine (DAB) as the chromogen. Two investigators confirmed the diagnosis of the specimens and evaluated the results qualitatively and quantitatively.

STATISTICAL ANALYSIS

QUALITATIVE ASSESSMENT

The pattern, intensity and distribution of IHC staining of all the test and control sections were evaluated using a light microscope under various magnifications up to 1000× (Leica DM DigitalMicroscope, CTR5000, Leica Microsystems, USA). The staining pattern was classified as cellular membrane, cytoplasmic or mixed cell membrane/cytoplasmic staining. The staining intensity was graded into three groups: strong and intense staining (50% or more of the cells stained), weak staining (less than 10% of the cells stained) or no staining.

QUANTITATIVE ASSESSMENT

Photomicrographs of each section were taken at ×400 magnification and viewed after fitting with a digitally superimposed graticule (calibrated grid measuring in total at 250 μm). A software package from MacBiophotonics (MBF) Image J for microscopy (Windows 32-bit version) was used to quantify cells and a specific schedule was followed. One thousand mononuclear immune cells in the fibrous connective tissue were counted for each slide. The total number of cells and the number of positively stained cells were recorded. Results were expressed as a percentage of the total number of immunopositive mononuclear immune cells per total number of mononuclear immune cells that were present. Immunostaining markers data was analysed and quantified using Kruskal-Wallis and Mann-Whitney U tests, with SPSS software Windows version 18.0 (SPSS Inc., Chicago, IL). A p value of less than 0.05 was considered statistically significant.

RESULTS

For the test groups, the mean age was 58 years (hyperplastic OMLP) and 54 years (atrophic OMLP); the youngest patient was 34 years and the oldest was 84 years. Most patients were female (3:2). A younger age distribution was seen in the control group (mean age 47 years, range 14 years to 67 years) with a similar female predominance (4:1).

QUALITATIVE ANALYSIS

Each IHC tissue section was first evaluated for the presence of reaction product (positive or negative), quality of the staining (staining pattern, intensity and distribution of positive cells) and background staining within the sections.

All positive control tissue sections showed positive staining against every immune cell marker type tested (CD3+, CD4+, CD8+, CD19+ and CD83+) (Figures 1(a), 1(d), 1(g), 1(j) and 1(m)) T lymphocyte cell subsets (CD3+, CD4+ and CD8+), B cell (CD19+) and mature dendritic cells (CD83+) showed intense immunostaining in OMLP with a higher number of positive cells than in the control group. All T cells (CD3+, CD4+ and CD8+) and B cells (CD19+) exhibited both cellular membrane and cytoplasmic staining although the membranous reaction was more apparent. Most mature dendritic cells (CD83+) showed distinct cytoplasmic staining even though both cell membrane and cytoplasm were immunostained. Distinct brown DAB chromogen staining was observed with each antibody type with intensity comparable to the positive control tissue. All T cell subsets (CD3+, CD4+ and CD8+) showed strong and intense staining in both test and control group. B cells (CD19+) demonstrated strong and intense staining in atrophic and hyperplastic OMLP. None of the CD19+ve B cell was stained in the control group. Mature dendritic cells (CD83+) in the test groups exhibited a strong and intense staining, however only weak staining was apparent in the control group.

In both OMLP subtypes, a diffuse T cell (CD3+ and CD4+) infiltrate was seen in the superficial connective tissue and scattered within the epithelium (Figures 1(b) and 1(e)). A similar distribution was observed with CD8+ cytotoxic T cells (Figure 1(h)). Two of the OMLP samples exhibited lymphoid follicle formation. The follicles stained positively for all T cell subsets (CD3+, CD4+ and CD8+) and were negative for B cells (CD19+) and mature dendritic cells (CD83+). B cells (CD19+) were only found localised and scattered within the cellular infiltrate in the superficial connective tissue (Figure 1(k)). Localised and sparsely distributed mature dendritic cells (CD83+) were seen in the superficial and deeper connective tissue in all OMLP samples (Figure 1(n)). In the control group, positive reactivity for all T cell subsets (CD3+, CD4+ and CD8+) was seen on scattered inflammatory cells in the epithelium and connective tissue (Figures 1(c), 1(f) and 1(i)). No positive B cells (CD19+) were seen in this group (Figure 1(l)). For mature dendritic cells (CD83+), only a few, scattered cells were seen in the connective tissue (Figure 1(o)). Table 1 summarizes each immune cell type staining pattern, intensity and distribution between the control and test group.

QUANTITATIVE ANALYSIS

CD3+ mature T cells were present in all groups, with a mean value of 46% in the control group and a mean value of 80% in the hyperplastic OMLP group. The mean value for both test groups (atrophic and hyperplastic OMLP) was in the range between 78% and 80%, indicating that CD3+ distribution did not differ significantly between different types of OMLP. Overall, the control group had the least CD3+mature T cells. CD4+ helper T cells were present in all groups with an ordinal mean value (highest to lowest)

of 52% (atrophic OMLP), 49% (hyperplastic OMLP) and 31% (control group). The control group had the fewest CD4+ cells. Fewer CD8+ cells (range 5% to 25%) were seen in the control group. There were 1.5 times fewer CD8+ cytotoxic T cells than CD4+ T cells in both the OMLP and the control groups. CD19+ B cells made up only a small percentage of the inflammatory cell population present in both the atrophic (3%) and hyperplastic (3%) OMLP groups and none was present in the control group. Of the immune cells assessed, CD83+ mature dendritic cells were present in the least number. Atrophic OMLP tissue had more CD83+ mature dendritic cells than the hyperplastic group and the control group.

In order to determine whether the immunohistochemical findings were significantly different between the OMLP and control groups, Kruskal-Wallis analysis was performed. All T and B cell markers were found to be statistically higher in both OMLP test groups (atrophic and hyperplastic) compared with the control group ($p<0.001$). Interestingly, CD83+ mature dendritic cells were present in greater numbers ($p<0.05$) in both OMLP groups compared with the control group (Table 2). To further define and assess the level of statistical significance across the categories of the tissue groups, the Mann-Whitney U (non-parametric test/2-independent groups) was used. It was found that there was no significant difference in the types of immune cells between atrophic OMLP and hyperplastic OMLP (Table 2). Therefore, all immune cells that were assessed showed no significant difference in distribution in the two forms of OMLP. There was a difference in the number of immune cells expressing the surface markers CD3+, CD4+, CD8+, CD19+ and CD83+ between the atrophic OMLP group and the control group with all showing statistically significant p values <0.05. When comparing hyperplastic OMLP and the control group, there were statistically significant differences for only the T and B cell markers ($p<0.001$). Differences in CD83+ expression were not statistically significant.

To further examine whether there was any relationship between the strength of linear dependence between variables, a bivariate correlation (Pearson correlation) analysis was performed (Table 3). All T cell subtypes (CD3+, CD4+ and CD8+) and B cells (CD19+) had a positive correlation close to 1; showing a direct and fairly strong relationship between these lymphocyte subsets, whereby as one type increases, the other subset increases ($p<0.01$) in both OMLP groups and the control group. Interestingly, CD83+ mature dendritic cells had only one direct and positive linear relationship with CD4 helper T cells ($p<0.05$).

DISCUSSION

OMLP is a relatively common chronic inflammatory mucocutaneous disorder that affects the population worldwide (Scully et al. 1998). The demographic distribution of our study showed similarities with those reported previously, where most patients were middle
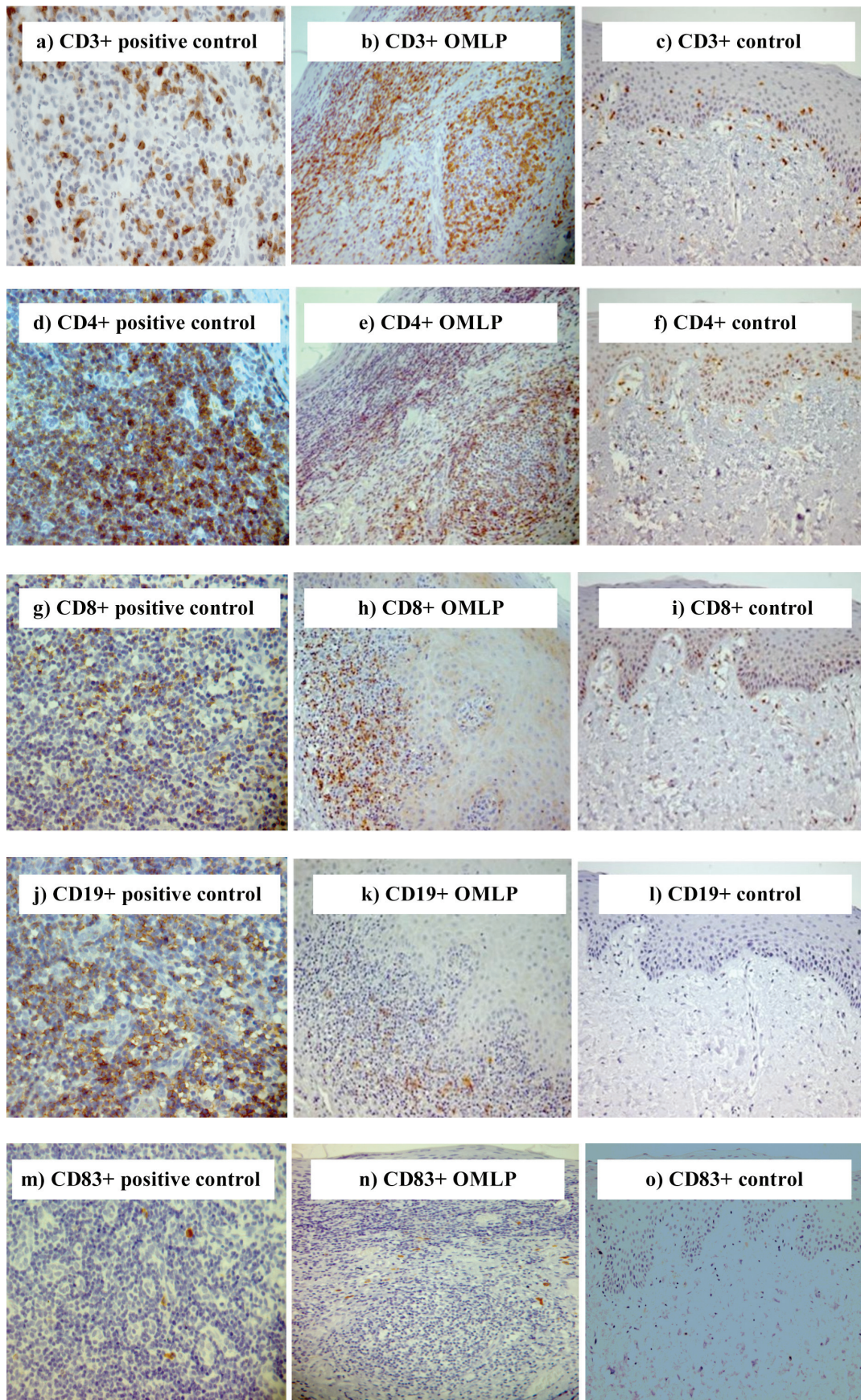
68

FIGURE 1. IHC Analysis of CD3+, CD4+, CD8+, CD19+ and CD83+ for positive control, OMLP and non-specific inflammatory control group (DAB) ×200 and ×400 magnification

TABLE 1. Summary of staining pattern, intensity and distribution between the control group and OMLP groups

Antibody/clone	All groups	Control group		OMLP groups	
Expression	Staining pattern	Intensity	Distribution	Intensity	Distribution
CD3/2GV6 (mature T cells)	Cellular membrane & cytoplasmic; predominately membranous staining	Strong, intense staining	Scattered in the epithelium and connective tissue	Strong, intense staining	Diffuse infiltration in the superficial connective tissue and scattered within the epithelium
CD4/SP35 (helper T cells)	Cellular membrane & cytoplasmic; predominately membranous staining	Strong, intense staining	Scattered in the epithelium and connective tissue	Strong, intense staining	Diffuse infiltration in the superficial connective tissue and scattered within the epithelium
CD8-α/ O.N.66 (cytotoxic T cells)	Cellular membrane & cytoplasmic; predominately membranous staining	Strong, intense staining	Scattered in the epithelium and connective tissue	Strong, intense staining	Diffuse infiltration in the superficial connective tissue and scattered within the epithelium
CD19/LE-CD19 (B cells)	Cellular membrane staining	No staining	No staining	Strong, intense staining	Localised and scattered in the superficial connective tissue
NCL-CD83/IH4 (mature dendritic cells)	Cellular membrane and cytoplasmic; predominately cytoplasmic staining	Weak staining	Scattered in the connective tissue	Strong, intense staining	Localised and scattered in superficial and more deeper connective tissue

TABLE 2. Non-parametric test for atrophic and hyperplastic OMLP and the control group

Immune cell marker	Atrophic OMLP % (sd)	Hyperplastic OMLP % (sd)	Control % (sd)
CD3^{+}	78 (9)[a*]	80 (6)[a*]	46 (9)
CD4^{+}	52 (8)[a*]	49 (8)[a*]	31 (13)
CD8^{+}	35 (9)[a*]	32 (8)[a*]	20 (11)
CD19^{+}	3 (3)[a*]	3 (3)[a*]	-
CD83^{+}	2 (1)[b**]	2 (1)[b]	1 (1)

[a]$p<0.001$, [b]$p<0.05$; Kruskal-Wallis Test
[*]$p<0.001$, [**]$p<0.05$; Mann-Whitney U Test

TABLE 3. Pearson correlation between associated variables

Immune cell marker	CD3^{+}	CD4^{+}	CD8^{+}	CD19^{+}	CD83^{+}
CD3^{+}	1	1[*]	1[*]	0.4[*]	0.2
CD4^{+}	1[*]	1	0.5[*]	0.4[*]	0.3[**]
CD8^{+}	1[*]	0.5[*]	1	0.4[*]	0.2
CD19^{+}	0.4[*]	0.4[*]	0.4[*]	1	0
CD83^{+}	0.3	0.3[**]	0.2	0	1

[*]$p<0.01$, [**]$p<0.05$; Pearson correlation analysis

aged females (female to male ratio; 3:2) (Eisen 2002; Sugerman & Savage 2002). Most OMLP samples were from the buccal mucosa (about 90% of cases), a common site involved in OMLP. This is followed by the tongue, alveolar ridge and gingiva although multiple sites may be involved (Axell & Rundquist 1987; Eisen 2002; Sugerman & Savage 2002).

Current studies show strong evidence that immunological mechanisms are involved in the pathogenesis of OMLP. Most authors support the idea that immune system allows proliferation and activation of T lymphocytes which are involved in OMLP (Porter et al. 1997; Sugerman et al. 2002; Thornhill 2001; Walsh et al. 1990). Our study showed that T lymphocytes (CD3+, CD4+ and CD8+), B lymphocytes (CD19+) and mature dendritic cells (CD83+) in OMLP had a significantly higher number of positive cells present than in the non-specific inflammatory control group. The present study also confirmed the dominance of T lymphocytes over B lymphocytes, as has been previously shown by other studies (Hirota et al. 1990; Jungell et al. 1989; Walsh et al. 1990). There was a positive correlation and direct relationship between these T and B lymphocyte subsets whereby as one type increased within the infiltrate in OMLP, the other subset follows. A higher proportion of CD4+ helper T lymphocytes were seen than CD8+ cytotoxic T lymphocytes in both hyperplastic and atrophic OMLP groups. This finding is concordant with earlier studies (Bhan et al. 1981; Boisnic et al. 1990; Dorrego et al. 2002) which relate cellular distribution with disease progression. In early lesions there was an influx of CD4+ helper T lymphocytes whereas in older OMLP there was a substantial increase in CD8+ cytotoxic T cells which were associated with membrane disruption (Porter et al. 1997; Walsh et al. 1990; Zhou et al. 2002). There was no significant difference in the distribution of types of immune cells between atrophic (erosive) and hyperplastic (reticular) OMLP groups. Our findings were in agreement with Charazinska-Carewicz et al. (2008) and Rodriguez-Nunez et al. (2001) where no statistically significant differences were observed in the peripheral blood T lymphocyte subsets Th1 cells in patients of both OMLP groups but contradicts with an earlier study where it was proposed that immune cell distribution in lichen planus correlated with the clinical form of the disease (Al-Fouzan et al. 1996).

In the non-specific inflammatory control group, T lymphocytes (CD3+, CD4+ and CD8+) and mature dendritic cells (CD83+) were present, but CD19+ B cells were absent. This suggests the possibility of T lymphocytes and mature dendritic cells play an important role in immune surveillance by monitoring the immune system for pathogen associated molecular patterns (PAMPs) or foreign substances, destroying the affected cells and coordinating the overall immune response (Walton et al. 1998). CD19+ B cells were present, but made up only a small percentage of the immune cell population in both OMLP groups. Interestingly, atrophic OMLP showed slightly more CD19+ B cells compared with the hyperplastic OMLP group, but this is not statistically significant. B cells and plasma cells are known to be low in numbers in OMLP lesions (Charazinska-Carewicz et al. 2008; Khan et al. 2003; Sugerman et al. 2002) and furthermore, OMLP patients do not have consistent serum immunoglobulin levels (Mahood 1981), suggesting a weak correlation with B cell associated Th2 response in OMLP (Porter et al. 1997).

Of the immune cells assessed, CD83+ mature dendritic cells were present in the least number and were mostly localized to areas in the connective tissue where there were lymphocyte aggregates, where they were thought to play an ongoing role in antigen presentation (Gustafson et al. 2007). Mature dendritic cells (CD83+) had only one direct relationship and that was with CD4+ T helper. Atrophic OMLP tissue had more CD83+mature dendritic cells than the hyperplastic group and the control group.

CONCLUSION

In conclusion, CD3, CD4 and CD8+ve T cells were the predominant inflammatory cell type in OMLP rather than CD19+ B cells, supporting the role of Th1 cells in the pathogenesis of OMLP. CD83+ mature dendritic cells were present in the least number and were mostly localized to areas where there were lymphocyte aggregates. There was a positive correlation and direct relationship between T and B lymphocyte subsets whereby as one subset increased, the other subset also increased.

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