Texila International Journal of Public Health
Special Issue-2024
DOI: 10.21522/TIJPH.2013.SE.24.06.Art012

Immunophenotyping Peripheral Blood Lymphocyte (TBNK) and beyond in Severe Periodontitis Reveals Immunodeficiency

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Abstract

Bacterial biofilms and host immune responses combine intricately to cause periodontitis, a chronic inflammatory disease that gradually destroys periodontal tissues. Here, notable changes in the subsets of lymphocytes were observed. The patient had increased counts of central memory T-helper cells and different B cell subsets, but significantly lower levels of both total T and B lymphocytes, with a drop in CD4+ T cells. This pattern points to a potential immunodeficiency that may worsen periodontitis by compromising the control of inflammation. A dysregulated immune response is additionally indicated by elevated pre- and post-germinal center B cell counts and naïve cytotoxic T cell counts, which may offset decreased T cell-mediated immunity. This study uses immunophenotyping by flow cytometry to obtain the patient's immunological profile and tried to find out any possible underlying immune dysfunctions in the context of severe recurrent periodontitis. The results underscore the plausible contribution of immunological dysfunction to the endurance and intensity of periodontitis and stress the necessity of tailored immunotherapies for enhanced management of persistent cases. The study's shortcomings, despite the thorough immunophenotyping, are that it only focused on one patient, who might not be a representative sample of the broader population, and it lacked longitudinal data to evaluate changes over time. The comprehensive immunological analysis that flows cytometry offers is where the strengths are. More extensive cohorts should be used in future studies to examine the effectiveness of immunomodulatory treatments in improving treatment outcomes for patients with severe periodontitis.

Keywords: *Central Memory T-Helper Cells, Chronic Periodontitis, Flow Cytometry, Immunodeficiency, Immunophenotyping, Lymphocyte Subsets.*

Introduction

Periodontitis is a chronic, multifactorial inflammatory condition that is brought on by the build-up of dental plaque (also known as dental biofilm or biofilm), and it is characterized by the gradual destruction of the structures that support the teeth, such as the periodontal ligament and alveolar bone [1, 2]. Complex dynamic interactions between bacterial infections, harmful host immune responses, and environmental variables like smoking are involved in the disease [1, 3]. Gingival inflammation, clinical attachment loss, radiographic evidence of alveolar bone loss, locations with deep probing depths, mobility, bleeding following probing, and pathologic migration are all common signs of periodontitis [2, 4]. Globally, 743 million people may have severe periodontitis, accounting for around 11% of the world's population [5, 6, 7]. Oral health-related quality of life (OHRQoL) may be significantly harmed by periodontitis and its clinical repercussions,

Received: 04.09.2024 ***Accepted: 13.10.2024*** ***Published on: 20.11.2024***
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including tooth loss, whereas effective treatment may enhance patients' OHRQoL [8, 9]. The colonization of mixed bacteria in the oral tissue is the main factor contributing to periodontal disease [10], while there are other elements that act as secondary etiologic factors hastening the spread and development of periodontal diseases, such as calculus, overhanging restorations, dental plaque, developmental grooves, cervical enamel projections, anatomical features like the short trunk, cervical enamel projections, genetic elements, smoking, systemic elements and stress [11,12]. Flow cytometry is a method that is used to analyze the antigenic expression on single cell or cells suspended in a buffered salt-based solution quickly. This is a powerful tool used in numerous fields including immunology, virology, molecular biology, cancer biology, and infectious disease for studying the immunophenotype of cells and the immune system and also to study how the immune system reacts to cancer and infectious diseases [13]. Since flow cytometry makes it possible to examine several parameters at once, quickly, and affordably, it has emerged as the preferred method for immune cell monitoring. This makes flow cytometry suitable for routine clinical application [14]. As a result, many cell subsets can be discovered; nevertheless, to effectively separate populations of interest, complicated conjugate antibody combinations must be established [15].Numerous fluorochromes can be found that absorb light of the same wavelength while individually emitting light of distinct, different wavelengths, this is the foundation of polychromatic flow cytometry, which enables the simultaneous reading of a flow cytometry sample labelled with various fluorochrome–antibody complexes with a single laser pass [16]. The technique of using fluorescently tagged antibodies to recognize and measure discrete cell subpopulations within a heterogeneous cell population is known as immunophenotyping; frequently, this term is more precisely used to describe the characteristics of immune system-related cell subsets [17]. Flow cytometry is the preferred method for immunophenotyping.

Following the evaluation of the severity of periodontitis, either non-surgical or surgical periodontal treatment can be pursued, non-surgical therapy will include use of mouthwashes, dentifrices, scaling and root planing and local drug use at the site of infection [13]. Surgical therapies include more invasive procedures such as periodontal flap surgery, guided tissue regeneration. If the condition is found in majority of the teeth, ultimately the solution would be to go for a full mouth rehabilitation.

Recurrent severe periodontitis is likely to be treated in the same way, but the reason as to why a patient has a recurring infection is currently not being investigated in routine dental check-ups and treatments. In the current study, we tried to investigate the reason for severe recurrent periodontitis in a patient through immunophenotyping. Immunophenotyping by flow cytometryis used to identify and classify the numerous immune cell subtypes found in a sample in suspension, such as blood or body fluids. Several analytical methods used for immunophenotyping inclues multicolor flow cytometry, hyperspectral flow cytometry, and mass cytometry. Among these techniques, multicolour flow cytometry is the most popular method. Thus, this study was undertaken to analyse the immune status of a patient with chronic periodontitis by immunophenotyping using multicolour flow cytometry.

Materials and Methods

Sample Collection: A peripheral blood sample of 1ml was collected in an EDTA vacutainer from a 55-year-old lady. This vacutainer was stored at room temperature until processing.

Sample Preparation: After collection, RBC lysis buffer (BD Biosciences, USA) was added to the sample and was incubated for 5-7 minutes

at room temperature. The sample was centrifuged at 1500 rpm for 5 minutes, and then washed twice with PBS remove the lysed RBC from the supernatant. 3-5µl of fluorescent labelled antibodies purchased from BD Biosciences; USA was used for this study. The following antibodies were used: CD4 IgM PerCP Cy5.5, CD45RA BV510, CD3 APC, CD16 and CD56 PE, CD27 BV421, CD45 APCH-7, CD19 PECy7, TCRγδ FITC, and lgD CD8. These antibodies were added to the WBC pellet and incubation was done at room temperature in the dark for 20 minutes. After incubation, centrifugation was performed a second time to separate the unconjugated excess antibodies from the sample. The cells were then resuspended in 500 µl PBS.

Flow Cytometry Analysis: 500 µl of resuspended cells were acquired using a flow cytometer (BD FACSlyric, BD Biosciences, USA). 1,000,000 events were acquired. The analysis was performed using FACSuite 4.1 software.

Results

The various plots obtained from flow cytometry analysis are shown in Figure 1. CD45 vs. side scatter plot in which the blue ring represents the total lymphocytes present in the sample, Figure 2 shows a CD3 vs. CD19 GD plot which shows the B and T cells - the red ring shows the total B cell population, the violet ring shows the TCR GD +ve and the pink ring shows the TCR GD -ve T cells. Figure 3 shows a CD3 vs CD4 plot highlighting the T-helper cells (green ring). Figure 4 shows a CD3 vs CD8 plot highlighting the cytotoxic T cells. Figure 5 shows a CD8 vs CD4 plot showing the switched germinal center B cells (purple ring) and the unswitched post germinal center B cells (red ring). Figure 6 shows a CD27 vs CD45 vs CD4, CD3 plot which shows the T-helper cell subsets. Figure 7 shows a CD27 vs CD45 vs CD8, CD3 plot which shows the T-cytotoxic cell subsets. Table 1 is a tabular representation of the data analyzed from all the plots mentioned above. Multiple abnormalities in lymphocyte subsets are revealed by the immunophenotyping data. With low absolute counts of T cells (1259 cells/µl) and B cells (77 cells/µl), the total lymphocyte count is substantially lower, suggesting a possible immunodeficiency. With a low count of 725 cells/µl, CD4+ T cells are most affected. However, the central memory fraction has an enhanced count of 934 cells/µl, which may indicate a response to repeated exposure to antigen. On the other hand, there is a noticeable increase in naïve CD8+ T cells (698 cells/µl), indicating a change in the composition of the T cell population. Notable alterations can also be seen in B cell subsets; higher numbers of pre- and post-germinal center (GC) B cells may indicate altered B cell response or maturation. These results call for additional research to identify the underlying immunological malfunction. Figure 8 shows the severity of periodontitis in the selected patient. When cross checked with the normal lymphocyte subset counts for a woman in her 50s, it was noticed that the total number of B and T cells and the total T-helper cells were down regulated, the central memory T-helper cells, the naive cytotoxic-T cells and all the B cell subsets are up regulated. This shows that the patient was in an immunocompromised state.

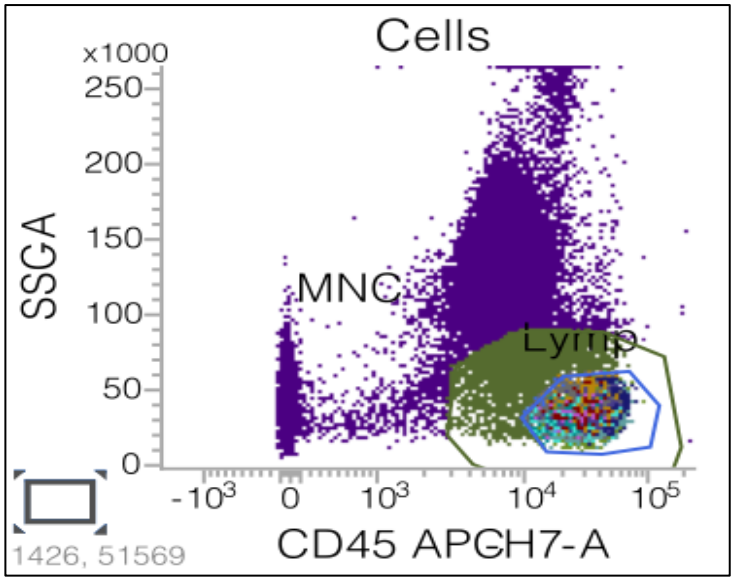

Figure 1: Is a CD45 vs side scatter plot highlighting the lymphocyte population

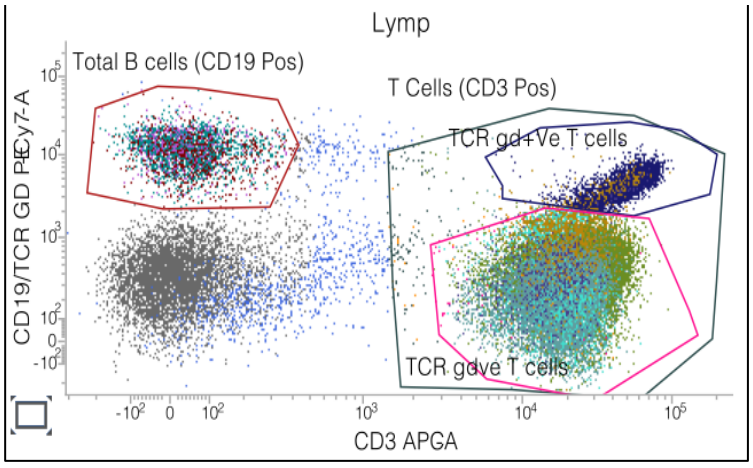

Figure 2. Is a CD3 vs CD19 TCR GD plot highlighting the B cells (red gated region), TCR GD -ve T cells (pink ring), and the TCR GD +ve T cells (violet ring)

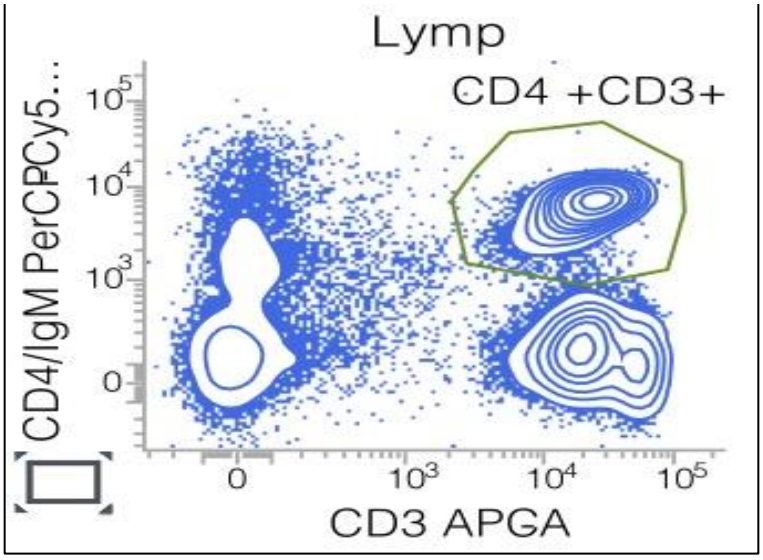

Figure 3: Is a CD3 vs CD4 plot highlighting the T-helper cells (green ring)

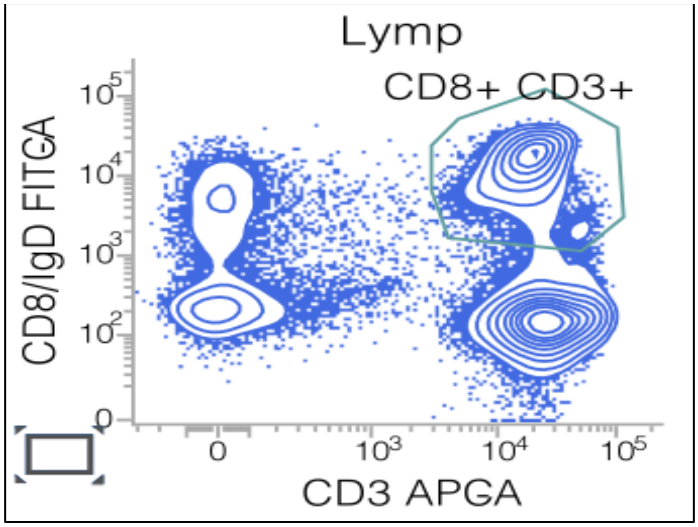

Figure 4: Is a CD3 vs CD8 plot highlighting the T-cytotoxic cells (light blue ring)

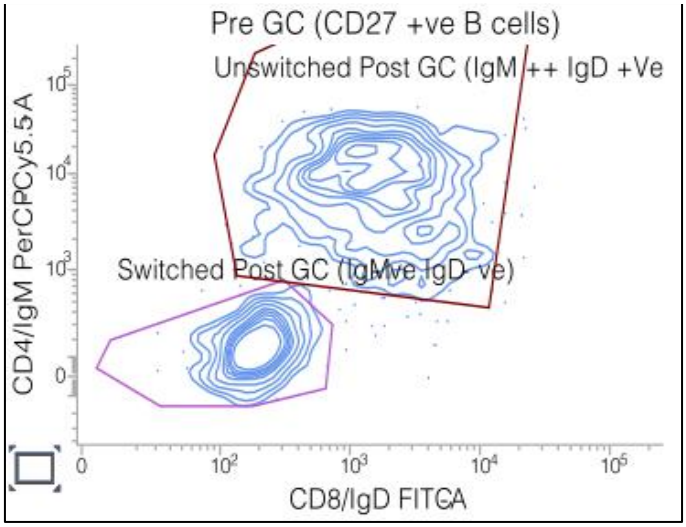

Figure 5: Is a CD8 vs CD4 plot highlighting the switched post germinal center B cells (purple ring) and unswitched post germinal center B cells (red ring)

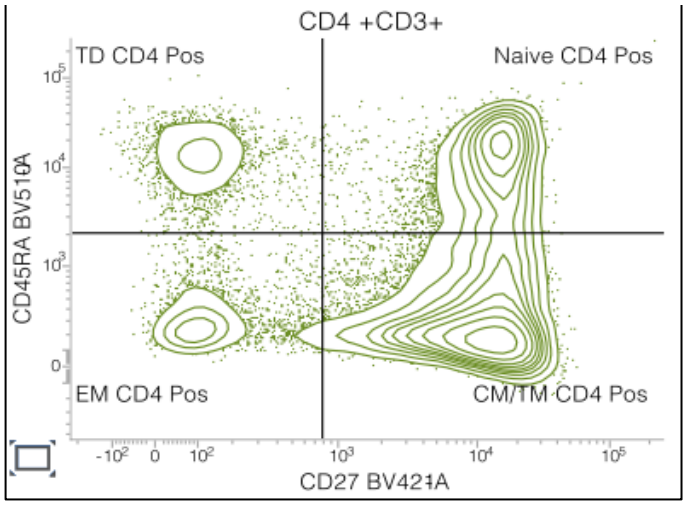

Figure 6: Is a CD27 vs CD45 vs CD4, CD3 plot showing T-helper cell subsets

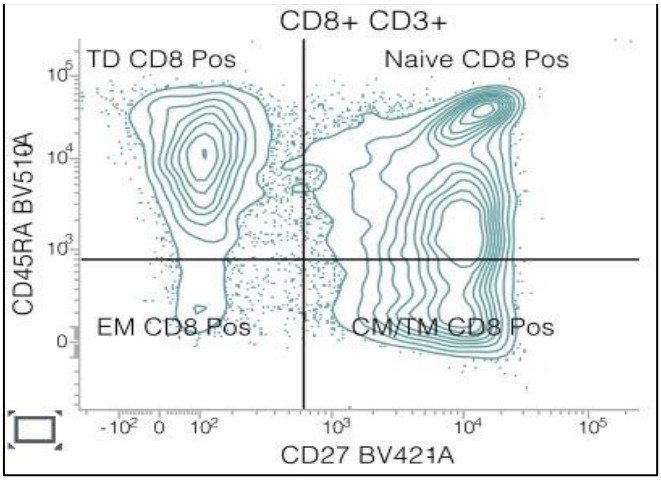

Figure 7: Is a CD27 vs CD45 vs CD8, CD3 plot showing T-cytotoxic cell subsets

Table 1. The Various Lymphocyte Cell Counts of the Patient

LYMPHOCYTE SUBSETS		
Lymphocytes	9.61	
T cells	**83.95**	**1259** ↓
B cells	**5.16**	**77** ↓
NK cells	10.3	155
CD4+T Cell subsets		
CD4+ T cells	**48.31**	**725** ↓
Naïve CD4+ T cells	24.34	365
Central Memory CD4+ T cells	**62.27**	**934** ↑
Effector Memory CD4+ T cells	7.09	106
Terminally Differentiated CD4+ T cells	6.29	94
CD8+ T CELL SUBSETS		
CD8+ T cells	24.45	367
Naïve CD8+ T cells	**46.54**	**698** ↑
Central Memory CD8+ T cells	26.09	391
Effector Memory CD8+ T cells	3.35	50
Terminally Differentiated CD27 dim CD8+ T cells	3.95	59
Terminally Differentiated CD8+ T cells	24.02	360
B CELLS SUBSETS (%)		
Pre GC B cells% (CD27+)	**33.5**	**502** ↑

Pre GC B cells% (CD27-)	**66.41**	**996 ↑**
Post GC unswitched B cells	**68.11**	**1022 ↑**
Post GC Switched B cells	**28.93**	**434 ↑**

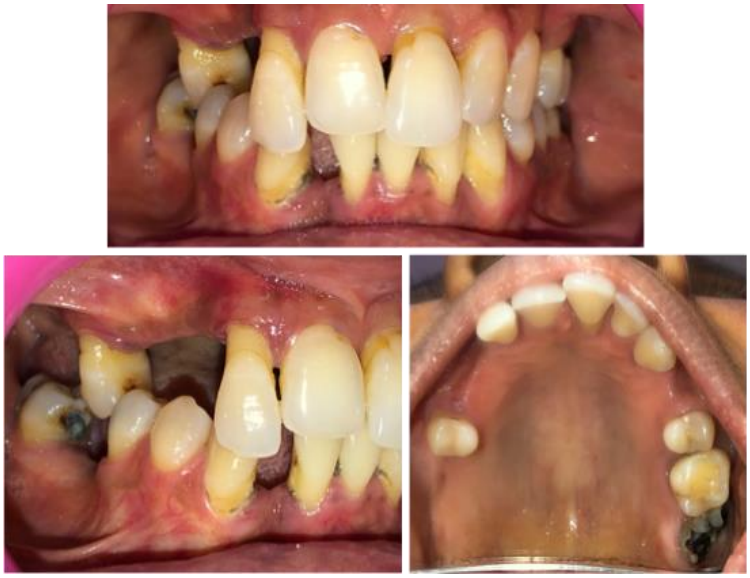

Figure 8: Intra oral images of the periodontitis patient

Discussion

A chronic inflammatory illness called periodontitis is typified by the breakdown of the tooth's supporting structures, which is mostly brought on by intricate interactions between bacterial biofilms and the host immune system [1, 10]. Studies indicate that abnormalities in immune cell subsets may add to the severity of the disease [18,19,20,21,22]. Immunological variables are important in the development and recurrence of periodontitis [23]. There are different in silico study to determine the role of the pathological conditions [24,25,26]. These immunological changes have been studied more and more with the use of flow cytometry, a potent instrument for examining immune cell profiles [13, 15]. This study used immunophenotyping to evaluate the immune status of a patient with severe recurrent periodontitis.

The findings showed a substantial change in the makeup of immune cells, as evidenced by a notable decline in the total numbers of T and B cells, especially CD4+ T cells. On the other hand, there was an increase of B cell subsets, naïve cytotoxic-T cells, and central memory T-helper cells, indicating an immunological response that might be connected to recurrent periodontitis. These results are consistent with other studies showing that immune cell distribution might be affected by chronic periodontitis, which may contribute to the persistence and advancement of the illness.

In a study by Naiff et al., [27] saliva samples of patients with chronic periodontitis were analysed through flow cytometry to investigate immune patterns, in the study, it was found that NK cells, CD4 T cells and B cell frequencies were found to be higher in the patient group when compared to the control group. These results align with this study where it was found elevated B cell subsets in the patient. A study conducted by Buduneli et al., found no significant difference between the relative counts of B cells, T cells and NK cells in the patient and control groups [18]. These differences imply that the immune reaction to periodontitis might be more nuanced and

situation-specific than previously thought. Certain immune cell modifications seen in various studies may be influenced by variables like the severity of the disease, the existence of co-existing systemic disorders, and even individual genetic predispositions. For example, afar et al. found that patients with adult periodontitis had a higher percentage of activated T lymphocytes, with raised levels of CD4+ and CD8+ T cells [28]. In contrast, the current study's considerable reduction in CD4+ T cells suggest a possible immunodeficiency rather than hyperactivation.

According to Zafiropoulos et al., individuals with advanced periodontitis showed changed T cell subsets, especially a reduction in CD4+/CD8+ ratios, which is frequently linked to long-term inflammatory diseases [29]. This result is consistent with the reduced number of CD4+ T cells found in this investigation, supporting the idea that periodontitis may be linked to an immune system that is weakened and unable to properly control inflammation. Walker also emphasized the acquisition of antibiotic resistance in the periodontal microbiota, implying that a chronic infection may be the cause of immune activation that persists and contributes to the changed immune cell profiles observed in patients with periodontitis [30]. Research like that conducted by Balaji et al. highlights the regulatory functions that T cell subsets play in preserving periodontal health [31]. They discovered that abnormalities in these subsets, like the rise in naïve CD8+ T cells observed in our investigation, may interfere with immunological homeostasis, and hasten the course of disease. In a similar vein, the rise in central memory T-helper cells that has been seen in this instance may indicate a continuous immunological challenge, possibly brought on by bacterial antigens that have persisted in the periodontal tissues.

The present study's observation of a shift towards central memory T-helper cells is consistent with previous research highlighting the critical function of memory-associated CD8+ T cells in the treatment of chronic periodontitis [32]. Further research has revealed that cases of severe periodontitis are associated with changes in B cell subsets, particularly those implicated in germinal center reactions. These findings are consistent with the increased pre- and post-germinal center B cells identified in this study [33]. There is a changes in the blood parameters in the oral disease patients [34,35]. Because abnormalities in both populations can result in either excessive tissue damage or insufficient immunological responses, the interaction between T and B cells is crucial in periodontal disease [36, 37]. There are immune disturbances in the chronic disease conditions and cancer [38-40]. Artificial materials such as calcium carbonate, PRF, and nano-hydroxyapatite, through their mineralization, have shown clinical advantages in multiple areas [41-43]. This study's increased B cell subsets could be an attempt to counteract the decline in T cell-mediated immunity, which could exacerbate the inflammatory response. The significance of immune profiling in comprehending the pathophysiology of periodontitis and peri-implantitis has been highlighted by recent research that use flow cytometry to investigate immune cells in these diseases. Treatment strategies may center on overlapping immunological pathways between periodontitis and peri-implantitis because the immune dysregulation observed in the current investigation is comparable to the altered leukocyte populations found in previous investigations [44].

Conclusion

Significant immunological alterations were found in this patient with severe recurrent periodontitis, including decreased T and B lymphocytes, particularly CD4+ T cells, and increased B cell subsets and central memory T-helper cells. These changes point to a possible immunodeficiency that may be causing problems with the regulation of inflammation,

which could make periodontitis worse. Pre- and post-germinal center B cells as well as naïve cytotoxic T cells are on the rise, which suggests a dysregulated immune response that may be compensating for decreased T cell activity. Targeted immunotherapies may help better control chronic periodontitis, according to the findings, which also emphasize the significance of immunological profile in this condition. However, the study's shortcomings include its singular patient focus, which could not be typical of the general population, and the absence of longitudinal data to evaluate immune status changes over time. The study's strength is its thorough immunophenotyping methodology, which offers insightful information about immunological dysfunction in periodontitis. Larger cohorts and the possibility of immunomodulatory therapies to enhance outcomes for individuals with severe periodontitis should be investigated in future studies.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

The authors are thankful for the support provided by Saveetha Dental College and Hospitals for the conduct of the study. and for the facilities provided by the Centre of Molecular Medicine and Diagnostics (COMManD), Saveetha University, Chennai.

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