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Flow cytometry helps differentiate between mucous membrane pemphigoid and erosive oral lichen planus

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This cross-sectional study investigated the immunophenotype of patients with mucous membrane pemphigoid (MMP) and erosive oral lichen planus (eOLP) evaluating flow cytometry as additional method in differential diagnosis. Thirty patients (18 MMP and 12 eOLP) were recruited. A blood sample was collected from each patient and B and T lymphocytes subsets were characterized through flow cytometry. Disease severity was additionally scored and MMP patients were characterized as high or low-risk based on the extent of mucosal involvement. Any correlation was finally investigated between the immunophenotype and clinical and serological data. High-risk MMP reported higher frequencies of memory cytotoxic T cells ($P < 0.01$), memory helper T cells ($P < 0.001$***), pre-switch and post-switch memory B cells ($P < 0.01$**) and lower frequencies of naïve cytotoxic T cells ($P < 0.01$**), naïve helper T cells ($P < 0.001$***) and naïve B cells ($P < 0.05$*) compared to low-risk MMP. Additionally, a significant lower frequency of Th1 cells ($P < 0.05$*) and memory cytotoxic T cells ($P < 0.001$***) was found in MMP compared to eOLP and both the biomarkers reported high diagnostic accuracy with an AU-ROC value of 0.76 ($P < 0.05$*) and 0.86 ($P < 0.001$***), respectively. No differences between the two diseases were reported in B-cell repertoire. T-cell immunophenotyping mirrors the different pathophysiology of MMP and eOLP and could provide a better understanding of the role of cellular immunity in MMP. MMP and eOLP can share similar clinical manifestations and can be challenging to distinguish through the histological and serological tests currently available. Flowcytometric assessment of T-cell subsets could be potentially useful in differentiating between the two diseases.**

Keywords Mucous membrane pemphigoid, Oral lichen planus, Flow cytometry, Immunophenotype, Differential diagnosis

Mucous membrane pemphigoid (MMP) is a group of autoimmune blistering diseases (AIBD) predominantly affecting mucous membranes and characterized by hemidesmosome-targeting autoantibodies[1]. The C-terminal epitope of bullous pemphigoid (BP)180 is the most recognized autoantigen (about 75% of patients), also in conjunction with BP230 (about 25%), while serum autoantibodies against other antigens such as laminin 332, integrin α6β4 and type VII collagen, are less frequently identified[2–4]. Clinically, most MMP patients (85%) report oral lesions with blisters, erosions and erythematous plaques typically located on attached gingiva and palate[5–7]. In severe cases, MMP manifests with a multisite involvement affecting conjunctiva, nasal mucosa, skin, anogenital area, pharynx, larynx and esophagus, in descending order of frequency[7]. A subepithelial blister associated with a polymorphic infiltrate is the most common, but non-specific histopathologic finding[8]. MMP diagnosis is based on direct immunofluorescence microscopy (DIF) of perilesional specimens reporting C3, IgG and/or IgA deposit along the dermal-epidermal junction (DEJ) and indirect immunofluorescence microscopy (IIF) on salt-split skin (SSS) showing IgG and/or IgA anti-basement membrane reactivity[9].

Oral lichen planus (OLP) is an inflammatory condition characterized by a T-cell-mediated immunological reaction to an unknow extrinsic antigen or altered self-antigen[10]. Hyperkeratotic Wickham's striae usually coalesce to form reticular, annular or plaque-like pattern and represent the distinctive clinical feature; bilateral buccal

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mucosa, borders and dorsum of the tongue and gingiva are the typically affected sites[10,11]. Nonetheless, OLP can manifest as an association of white striae and erythematous, erosive and ulcerative lesions[11,12]. Histopathological examination commonly shows a homogeneous band-like infiltrate of lymphocytes and histiocytes along the DEJ associated with liquefactive degeneration of epithelial basal cells and a saw-tooth pattern of epithelial rete ridges[13]. DIF is usually negative, but Civatte's bodies are coated by fibrinogen and can be detected as a shaggy linear fluorescence along the basement membrane[14].

Differential diagnosis between the MMP and OLP can be challenging. When OLP has predominant erosive or ulcerative phenotype could be misdiagnosed with AIBD[15]. Furthermore, lichenoid changes have been reported to occur in patients with MMP and the term lichen planus pemphigoides (LPP) has been coined to designate the association of clinical and histologic features of LP and AIBD of the pemphigoid group, including MMP[16]. Eighty-three cases of LPP affecting mucous membranes have been described so far. Among these patients, 80 (96.4%) reported oral lesions and the other involved sites were skin (79.5%), genitals (26.5%), nasopharynx (8.4%), eyes (4.8%) and esophagus (1.2%)[17]. A diagnosis of LPP is made when Wickam's striae and blisters are clinically evident, and histology shows both band-like lymphocytic infiltrate with hydropic degeneration of basal cells and subepithelial clefting[18]. DIF is positive revealing C3 and IgG deposition along the DEJ and in 50% of cases IgG presence is confirmed through IIF on SSS[10,19].

Therefore, many authors suggest to perform immunofluorescence, ELISA and immunoblotting studies in all cases of uncertain diagnosis to differentiate between OLP and MMP[20,21]. However, most of the tests mentioned are costly and are not widely available for routine diagnostic procedures[22]. Moreover, in MMP patients a false negativity is frequently obtained by DIF, making two or more biopsies needed to establish a final diagnosis, and IIF has a low sensitivity as circulating IgG titers are usually below 1:40[23–25]. Similarly, ELISA can be negative too as the commercially widely available tests recognize only BP180-NC16A and BP230-targeting autoantibodies and other autoantigens can be involved in MMP[4]. Thus, novel biomarkers to use in differential diagnosis are currently needed.

Flow cytometry has been increasingly used in the diagnosis of autoimmune diseases and in the management of patients as it provides a multiparametric evaluation of the phenotypes and functional status of the immune system[26,27]. Furthermore, this technique has gained substantial relevance in autoimmunity research, as it allows to investigate the interactions among cellular subsets, thereby providing novel insights into the immunopathogenesis of autoimmune diseases and supporting the development of novel targeted therapies[28].

This study aimed to evaluate the cytofluorimetric profile of patients with erosive OLP (eOLP) and MMP to assess flow cytometry accuracy in differentiating between these diseases. Any correlation was additionally investigated between B and T-cell immunophenotypes and patients' clinical and immunological characteristics.

Materials and methods

Patients

This cross-sectional study was conducted according to the World Medical Association Declaration of Helsinki. Approval was obtained by the Ethical Committee of the University of Naples Federico II and written informed consent was signed by each participant. A total of 30 patients were recruited at the Oral Medicine Unit of the University of Naples Federico II between November 2023 and March 2025. A diagnosis of MMP was confirmed by (1) clinical evidence of erosive, ulcerative or bullous lesions, (2) mucosal histopathology revealing a subepithelial clefting, (3) perilesional DIF demonstrating IgG, IgA and/or C3 continuous deposits along the DEJ or ELISA (EUROIMMUN, Lubeck, Germany) detecting serum autoantibodies against BP180 and/or BP230[9]. The following clinical and histopathological criteria were used to diagnose eOLP: (1) presence of keratotic striations associated with erosions, ulcerations or blisters, (2) hematoxylin and eosin staining revealing a lympho-histiocytic band-like infiltrate and basal-cell liquefactive degeneration in combination with subepithelial blisters, (3) DIF and ELISA failing to detect tissue-bound and circulating antibodies targeting hemidesmosome antigens[29]. Figure 1 shows MMP and eOLP clinical features. Patients were excluded if (1) were affected by any neoplastic or immune-mediated comorbidity, (2) had received a previous immune-modulating therapy, (3) had dental restorations that could suggest a diagnosis of oral lichenoid contact lesions or (4) reported a clear temporal association between the onset of the oral manifestations and the starting administration of a medication that could potentially trigger MMP or oral lichenoid drug reactions.

Clinical activity was scored through the Mucous Membrane Pemphigoid Area Index (MMPDAI) for MMP[9], while OLP were clinically assessed using the Oral Disease Severity Score (ODSS)[30]. MMP patients were classified in high and low risk according to the extent of mucosal involvement[31].

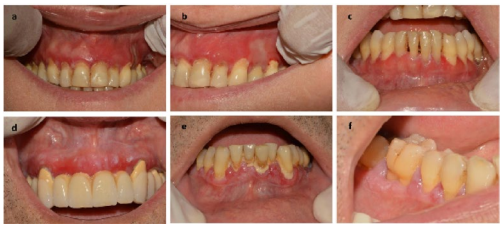

Fig. 1. Clinical characteristics of MMP and eOLP. (**a**–**c**) Erosions and ulcerations of attached and free gingiva in a patient with MMP. (**d**–**f**) Erosions and keratotic lesions of attached and free gingiva in a patient with eOLP. MMP, mucous membrane pemphigoid; eOLP, erosive oral lichen planus.

Flow cytometry

A blood sample was collected from each patient, and the phenotype of peripheral blood mononuclear cells (PBMCs) was determined using the following procedure. An aliquot (50 µL) of anti-coagulated ethylenediaminetetraacetic acid (EDTA) whole fresh blood (within 12 h) was incubated at 4 °C for 30 min in the presence of appropriate amounts of monoclonal antibodies, ensuring that the process was carried out in the dark. The mixtures were then diluted 1:20 in *BD Pharm Lyse*™ lysing solution (from BD San Diego, CA, USA), incubated at room temperature for 10 min, and finally washed before flow cytometric acquisition. For the determination of immunoglobulins, the samples were thoroughly washed before incubation with monoclonal antibodies to remove excess serum Ig. Acquisition was performed using the DxFlex cytometer (Beckman Coulter, Marseille Cedex 9, France), and data analysis was carried out afterward using CytExpert software (Beckman Coulter, Marseille Cedex 9, France). The following antigens were analyzed: CD4 PE (from BD San Diego, CA, USA), CD8 APCcy7 (from Beckman Coulter, Marseille Cedex 9, France), CD20 FITC (from BD San Diego, CA, USA), CD19 APC (from Beckman Coulter, Marseille Cedex 9, France), CD45 PerCP (from BD San Diego, CA, USA), CD27 HV500 (from BD San Diego, CA, USA), CD3 Pacific Blue (from Beckman Coulter, Marseille Cedex 9, France), CD38 Pecy5 (from Thermo Fisher Scientific Waltham, MA, USA), CD 127 FITC (from BD San Diego, CA, USA), CD25 PEcy7 (from Sony Biotechnology, San Jose, CA, USA), CD45RA APC (from Miltenyi Biotec, Bergisch Gladbach, Germany), CD45RO PE (from Miltenyi Biotec, Bergisch Gladbach, Germany), CD183 PE (from BD San Diego, CA, USA), CD196 PEcy7 (from BD San Diego, CA, USA), IgD PE (from BD San Diego, CA, USA), IgM APC (from Miltenyi Biotec, Bergisch Gladbach, Germany), CD24 APCcy7 (from Beckman Coulter, Marseille Cedex 9, France). The lower level of detection was 10^{-4} (thus, zero corresponds to a level below 1/10,000 cells). The gating strategy was as follows: lymphocyte cells were gated using CD45 vs. SSC-A, identifying 100,000 events. Values were expressed as percentages. Doublets and debris were excluded and removed by performing a Forward Scatter Area (FSC-A) vs. Forward Scatter Height (FSC-H) plot within the leukocyte gate. All laboratory procedures were conducted in accordance with UK-NEQAS quality standards (https://ukneqas.org.uk/).

Statistical analysis

Mean ± SD were used for descriptive statistics. Normality and variance homogeneity were evaluated through Shapiro-Wilk and Levene test, respectively. Gender distribution was evaluated using the Fisher's Exact test while the Independent-samples T test and the Mann Whitney U analysis were performed to compare normal and non-normal distributions, respectively. Pearson's and Spearman's coefficient were additionally used to assess any correlation between cellular frequencies and patients' clinical and serological data. To investigate flow cytometry diagnostic accuracy the receiver-operating characteristic curve (ROC) was made and area under the curve (AUC) was calculated. All conducted tests were two-sided and p-value ≤ 0.05 (*), ≤ 0.01 (**) or ≤ 0,001 (***) were considered statistically significant. SPSS 29.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 10.4.0 (GraphPad Software, Boston, Massachusetts USA) were the software used for statistical analysis and graphing.

Results

Demographic and clinical characteristics

Characteristics of our cohorts are shown in Table 1. Twelve patients received a diagnosis of eOLP while 18 were diagnosed as MMP. Age and gender were homogeneously distributed among groups. All the patients reported upper and lower gingiva involvement with bullous or erosive lesions and, limited to eOLP group, gingival erosions were associated with keratotic striations. Other oral sites were affected in two MMP and two eOLP patients: one MMP reported erosive lesions involving the posterior hard palate while in another patient bilateral buccal fornices were involved; one patient with eOLP had white reticulations with perilesional erythema on bilateral buccal mucosa and the other one reported a wide erosion on the left buccal mucosa. Nine MMP had a high-risk disease with involvement of the oral cavity and conjunctiva, nasopharynx, larynx and/or genitals while skin involvement was reported in six patients. DIF and ELISA for BP 180-targeting antibodies were positive in 15 and ten MMP patients, respectively.

		MMP ($n=18$)	eOLP ($n=12$)	
Age (mean ± SD)[a]		62.7 ± 11.6	66.2 ± 7.8	ns
M: F (n)[b]		1:2.6	1:5	ns
MMPDAI (mean ± SD)		13.3 ± 9.8	–	
ODSS (mean ± SD)		–	9.25 ± 3.5	
Involved area (n and %)	Oral	18 (100)	12 (100)	
	Cutaneous	5 (28)	–	
	Ocular	3 (17)	–	
	Laryngeal	3 (17)	–	
	Genital	2 (11)	–	
	Nasopharyngeal	1 (8)	-	

Table 1. Characteristics of our cohorts. [a]Independent-samples T test. [b] Fisher's Exact test.

Correlation between clinical and immunological phenotypes

An association was reported in MMP between patients' clinical phenotype and B and T-cell subpopulations. High-risk MMP reported higher frequencies of memory cytotoxic T cells ($P<0.01$**), memory helper T cells ($P<0.001$***), pre-switch and post-switch memory B cells ($P<0.01$**) and lower levels of naïve cytotoxic T cells ($P<0.01$**), naïve helper T cells ($P<0.001$***) and naïve B cells ($P<0.05$*) compared to low-risk MMP (Fig. 2). Th1 and Th17 cells were, instead, similarly distributed among the groups. No correlation was found between MMPDAI value and flow cytometric immunophenotypes. Patients with detectable levels of anti BP-180 antibodies and those with negative ELISA had comparable values of B and T cells and no correlation was observed between autoantibodies titers and flow cytometric immunophenotypes. Contrarily to MMP, in eOLP patients no association was reported between disease clinical severity and B and T-cell subsets.

Flow cytometric immunophenotyping in MMP and eOLP

B cell subpopulations had similar frequencies in MMP and eOLP patients and no differences were reported between groups in total B cells and naïve, memory and class switched memory B-cell subsets. When evaluating T-cell immunophenotypes, total helper and cytotoxic T- cell percentages were comparable between groups, as well as naïve and memory helper T-cell and Th17-cell concentrations. MMP reported significantly lower frequencies of memory cytotoxic T cells ($P<0.001$***) and corresponding higher levels of naïve cytotoxic T cells ($P<0.001$***) compared to eOLP. Additionally, lower frequencies of Th1 cells were reported in MMP compared to eOLP ($P<0.05$*). (Fig. 3)

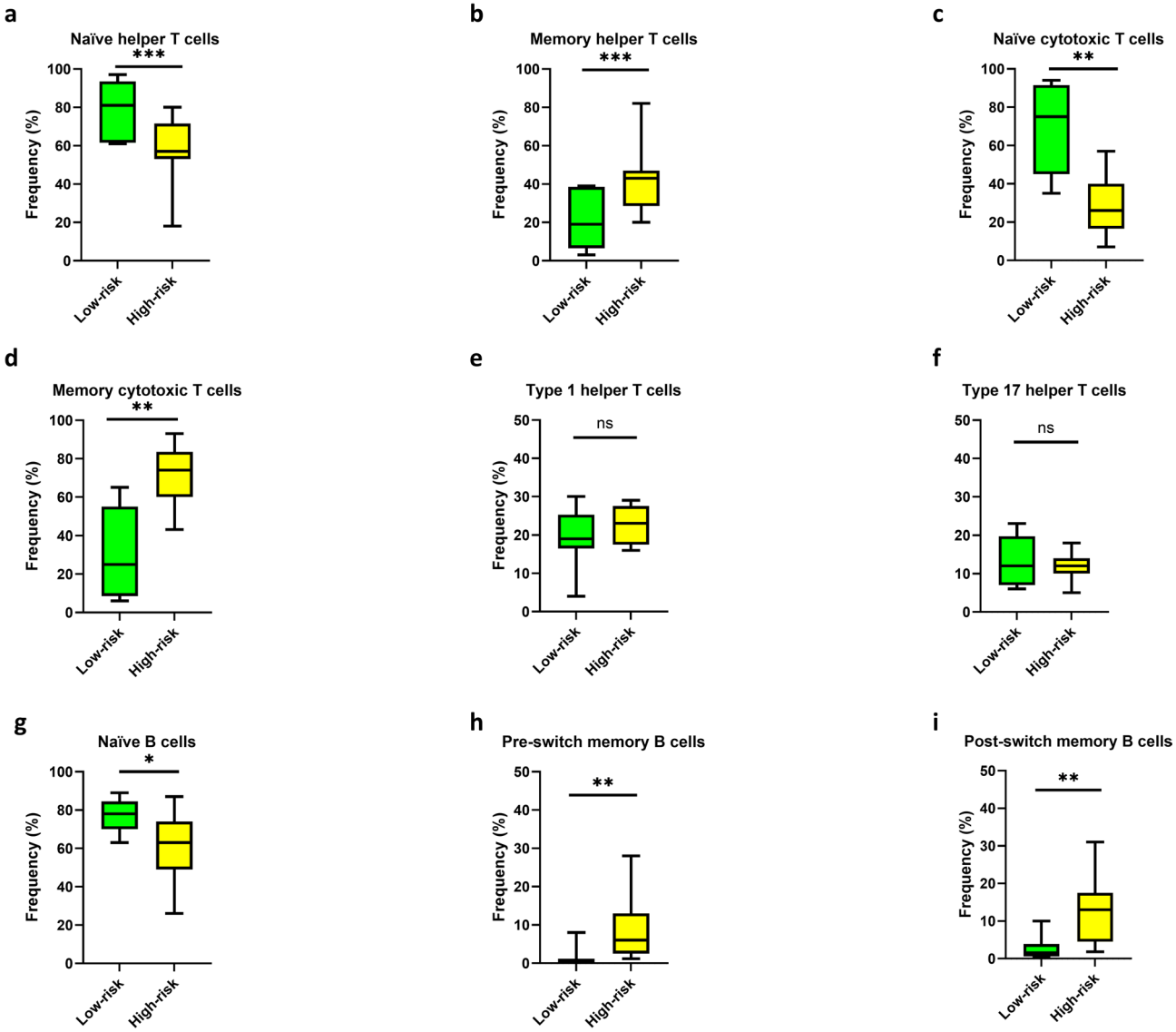

Fig. 2. Correlation between clinical phenotype and flow cytometric analysis in MMP. (**a**–**f**) T cells distribution among groups. (**g**–**i**) B cells distribution among groups. The independent-samples T test and the Mann Whitney U test were the statistical tests used for comparison. Statistical significance is demonstrated as p-values (*$P<0.05$, **$P<0.01$, ***$P<0.001$). ns, non-significant.

When evaluating the diagnostic efficacy of flow cytometry in differentiating between MMP and eOLP AUROC curve were 0.86 ($P < 0.001$***) and 0.76 ($P < 0.05$*) for memory cytotoxic T cells and Th1 cells, respectively. Memory $CD8^+$ cells reported the highest accuracy with a cut-off of 45% associated with a sensitivity and a specificity of 80%. For Th1 cells, instead, a sensitivity of 80% and a specificity of 70% were found at a cut-off of 21.5%. (Fig. 4)

Discussion

OLP and MMP are characterized by distinct pathophysiology as the first is a delayed-type hypersensitivity reaction while the latter is primarily an Ig-mediated condition. In OLP cytotoxic T cells are thought to trigger keratinocytes apoptosis via TNF-α and Fas-Fas ligand mechanisms after interacting with foreign antigen engaging-helper T cells through a "request cytotoxic activity" cell surface molecule, expressed both by $CD8^+$ and $CD4^+$ T lymphocytes[32]. In MMP, instead, autoantibodies bind to different BMZ antigens disrupting BP180 molecular interactions and leading to blister formation through a complement-independent mechanism[33].

Despite the different pathogenic mechanisms OLP and MMP can share similar clinical features and can be difficult to distinguish. This is especially true when patients have gingival involvement alone as gingival OLP is typically characterized by desquamative gingivitis with erythematous and erosive lesions in the absence of white striations and desquamative gingivitis is the most frequently reported feature of mild/moderate MMP[16,34].

Nonetheless, an accurate diagnosis is fundamental to establish appropriate patients' management. These conditions, in fact, not only have different treatments but also carry dissimilar prognostic risks as MMP, differently from eOLP, manifests with a scarring phenotype potentially leading to blindness, respiratory distress

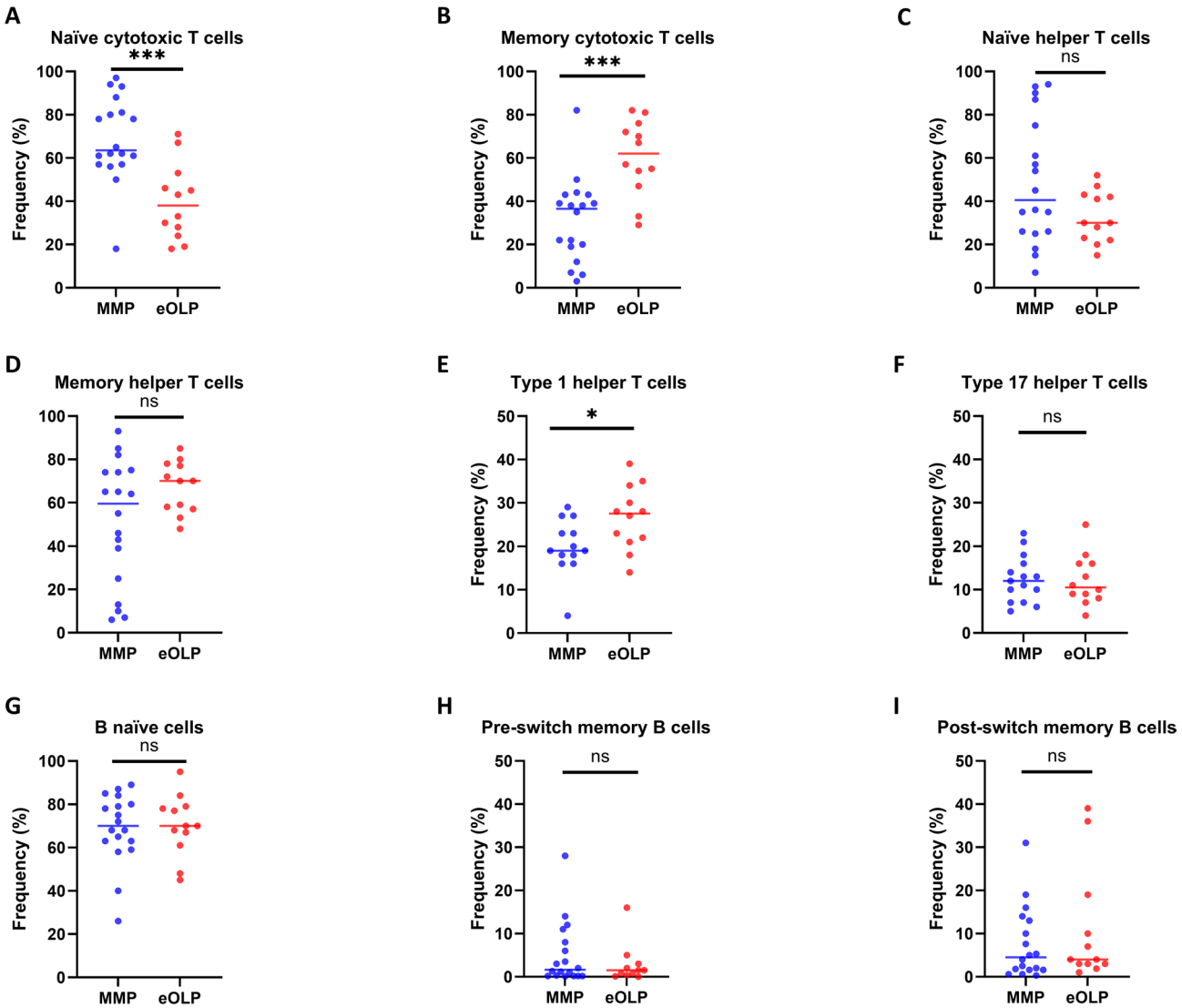

Fig. 3. Flow cytometric analysis of MMP and eOLP. (**a**–**f**) T cells distribution among groups. (**g**–**i**) B cells distribution among groups. The independent-samples T test and the Mann Whitney U test were the statistical tests used for comparison. Statistical significance is demonstrated as p-values (*$P < 0.05$, ***$P < 0.001$). ns, non-significant; MMP, mucous membrane pemphigoid; eOLP, erosive oral lichen planus.

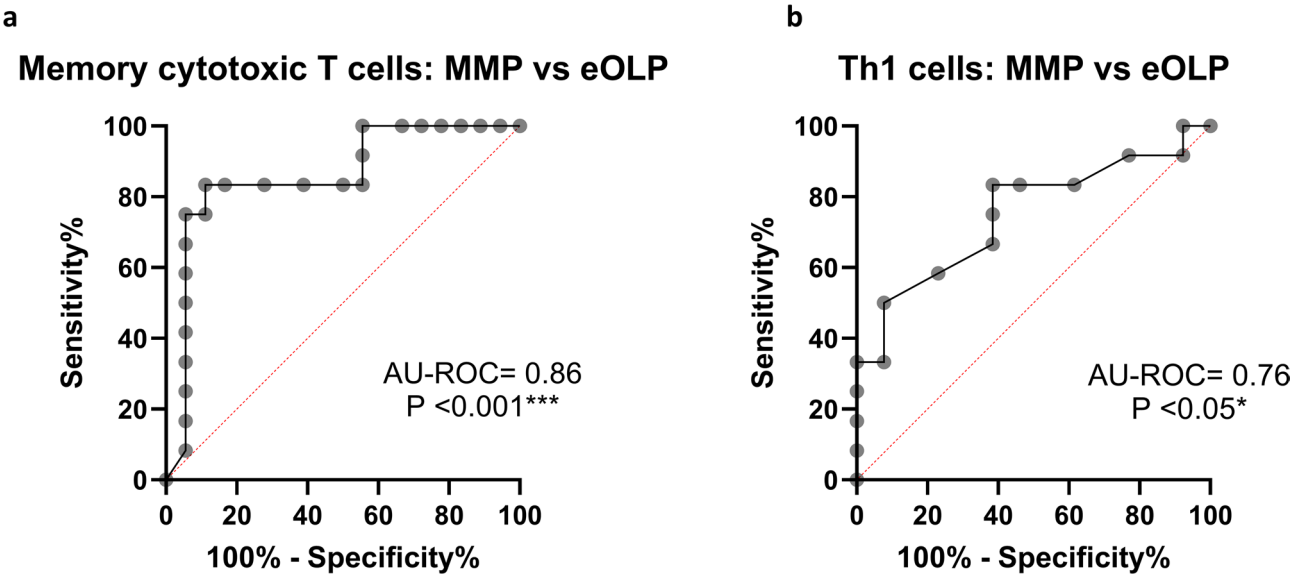

Fig. 4. Accuracy of memory cytotoxic T cells (**a**) and Th1 cells (**b**) in differential diagnosis between MMP and eOLP. AU-ROC analysis was the sensitivity test used. Statistical significance is demonstrated as p-values (*$P < 0.05$, ***$P < 0.001$). MMP, mucous membrane pemphigoid; eOLP, erosive oral lichen planus; AU-ROC, area under receiver-operating curve.

and severe dysphagia in patients with ocular, laryngeal and esophageal involvement, respectively[35]. At the same time eOLP, unlike MMP, is classified among the oral potentially malignant disorders, needing a close follow-up to reduce the risk of oral squamous cell carcinoma[36,37].

In this study, we investigated whether the flow cytometric assessment of PBMC could mirror the different pathogenesis of eOLP and MMP and could be an adjuvant tool in differential diagnosis. We found that eOLP patients have significantly higher frequencies of Th1 and memory cytotoxic T cells compared to MMP. Furthermore, both the lymphocyte subsets showed good sensitivity and specificity in the differential diagnosis with high AU-ROC curve values. These findings are in line with previous studies. Memory cytotoxic T cells were found to be significantly increased in OLP compared to healthy individuals and when stratifying eOLP patients based on their clinical phenotype higher values of $CD45RO^+$ lymphocytes were described in eOLP in comparison with patients with reticular lesions alone[38,39]. Moreover, a predominant type 1 T-cell mediated inflammatory response has been described in OLP. IFNγ/IL-4 ratio was found to be elevated both in serum and lesional tissue and Th1-induced expression of CCR5 was reported to be increased in OLP patients' T cells[40]. Similarly in cutaneous lichen planus a type 1 inflammatory signature was described in skin infiltrate in contrast to pemphigus vulgaris and bullous pemphigoid patients who reported a predominant type 2 T-cell response[41].

The role of T cells in MMP pathogenesis has not been elucidated yet. Nonetheless, $CD8^+$ and $CD4^+$ T cells were found to infiltrate conjunctiva in active ocular MMP and a high expression of IL-2 and MHC class II was reported in diseased tissue[42,43]. Moreover, in a minority of patients with MMP, NC16A-reacting T cells reported a rapid effector function and a correlation has been confirmed between MMP and HLA class II haplotypes with increased disease susceptibility reported in HLA-DQβ1*0301 carriers[44–46]. Interestingly, in this study higher values of memory B cells and T cells were found in patients with a more severe clinical phenotype supporting helper T-cell role in perpetuating autoantibodies production. In agreement with our results, higher HLA-DQβ1*0301 frequency was found in MMP patients with ocular involvement and higher clinical activity scores[44]. Moreover, in pemphigus vulgaris increased tissue-resident memory helper T cells positively correlated with disease severity and refractoriness and the same correlation has been demonstrated in other autoimmune diseases as higher memory T cells were found to be associated with increased Multiple Sclerosis Severity Score (MSSS) and Systemic Lupus Erythematosus Disease Activity Index- 2000 (SLEDAI-2k) in multiple sclerosis and systemic lupus erythematosus, respectively[47–49].

A limitation of our study is the small sample that could potentially limit the impact of our findings. Nonetheless, we recruited a group of MMP and eOLP with different clinical features, including both mild and severe disease and both oral and multiple mucosal or cutaneous involvement, then our sample could be considered fairly representative of the general population. Despite the small sample size multiple comparisons were made, in the absence of a correction method, potentially weakening the robustness of our results. This was, however, in line with the exploratory intent of our investigation that aimed to guide future research on this topic. To the best of our knowledge this is the first study to comprehensively investigate T-cell and B-cell repertoire in MMP and eOLP. We assessed lymphocyte subsets in peripheral blood and no evaluation of intralesional infiltrate was performed. Furthermore, the lack of a healthy control group could eventually preclude definitive conclusions on whether or not the observed immunophenotypic differences are specific to MMP and eOLP. Nonetheless, to increase the validity of our results, strict exclusion criteria were used in sampling and patients

with any neoplastic or immune-mediated disease that could potentially influence PBMC immunophenotyping were not included in the study.

In conclusion, we reported a correlation between cellular repertoire and patients' clinical characteristics in MMP. Additionally, T- cell immunophenotyping was found to differ between eOLP and MMP potentially mirroring the different pathophysiology and flow cytometry was suggested as adjuvant tool in differential diagnosis. Future studies with larger samples are needed to confirm our findings.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Received: 19 November 2025; Accepted: 21 January 2026
Published online: 27 January 2026

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Author contributions

Author contribution: Simone Liguori: Conceptualization, Formal analysis, Investigation, Writing- Original draft, Dario Didona: Validation, Writing-Review & Editing, Elvira Ruoppo : Investigation, Resources, Antonia Fiore: Investigation, Writing- Original draft, Giulia Scalia: Methodology, Resources, Michele Davide Mignogna: Conceptualization, Supervision, Stefania Leuci: Methodology, Supervision, Writing-Review & Editing.

Declarations

Consent to participate

Written informed consent was provided by each patient taking part in this study.

Ethics approval

This study was conducted according to the World Medical Association Declaration of Helsinki. Approval was obtained by the Ethical Committee of the University of Naples Federico II (protocol number 69/19).

Competing interests

The authors declare no competing interests.

Additional information

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