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Immunohistopathological characterization and the impact of topical immunomodulatory therapy in oral chronic GVHD: a pilot study

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Abstract

OBJECTIVE—To characterize the immunohistopathologic features of oral chronic graft-versus-host disease (cGVHD), and the impact of topical immunomodulatory therapy on the infiltrating cells.

MATERIAL AND METHODS—Paired oral cGVHD biopsies obtained before (n=12) and one month after treatment (n=12) with topical dexamethasone (n=8) or tacrolimus (n=4) were characterized by immunohistochemistry using a panel of CD1a, CD3, CD4, CD8, CD20, CD31, CD62E, CD103, CD163, c-kit and FoxP3. Controls included acute GVHD (aGVHD; n=3), oral lichen planus (OLP; n=5) and normal tissues (n=5).

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Conflict of interest

The authors declare no competing financial interests.

Author contributions

A.C.F.M. contributed to the study design, samples analyses, interpretation of data, and drafted the manuscript; R.J.S contributed to the clinical data; M.L. and S-B.W. contributed to the histopathological analyses; Q.Z. and A.L. contributed to immunohistochemistry analyses; G.F.M, R.J.S. and N.S.T contributed to the study design, data analyses, and drafted the manuscript; and all authors contributed to analysis and interpretation of data and critical review of the manuscript.

RESULTS—Oral cGVHD specimens prior to treatment were mainly characterized by basal cell squamatization, lichenoid inflammation, sclerosis, apoptosis, and lymphocytic exocytosis. The infiltrating cells in oral cGVHD primarily consisted of CD3⁺, CD4⁺, CD8⁺, CD103⁺, CD163⁺ and FoxP3⁺ cells, which were higher than in normal tissues. Topical dexamethasone or tacrolimus reduced neutrophilic exocytosis, basal cell squamatization and lichenoid inflammation in oral cGVHD, and dexamethasone reduced the number of CD4⁺ and CD103⁺ cells.

CONCLUSION—The high expression of CD3, CD4, CD8, CD103, CD163 and FoxP3 confirms that oral cGVHD is largely T cell driven with macrophage participation. The impact of topical immunomodulatory therapy was variable, reducing histological inflammatory features, but with a weak clinicopathological correlation. Topical dexamethasone reduced the expression of CD4 and CD103, which may offer novel therapeutic targets.

Keywords

Chronic graft-versus-host disease; oral mucosa; biomarkers; topical therapy

Introduction

Chronic graft-versus-host disease (cGVHD) is a serious complication of allogeneic hematopoietic cell transplantation (HCT) that affects approximately 25% to 80% of long-term survivors and is the leading cause of non-relapse mortality (Lee et al., 2003, Arai et al., 2011). Nearly 80% of patients with cGVHD present with oral involvement, making the oral cavity one of the most frequently affected target organs and a significant source of patient morbidity (Flowers et al., 2002, Arai et al., 2011). Oral mucosal disease is characterized by lichenoid inflammation presenting with hyperkeratotic white striations and plaques, erythema and ulcerations, with symptoms including oral pain, food intolerance and sensitivity, and a long-term increased risk of oral squamous cell carcinoma (Treister et al., 2013). Management is directed to minimizing symptoms and improving function, and often requires intensive localized ancillary therapy with topical immunomodulatory agents (Carpenter et al., 2015).

The pathogenesis of cGVHD remains poorly understood. It is generally recognized that the development of cGVHD involves a derangement of host immune function and cytotoxicity initiated by donor lymphocytes, allowing for the development of alloimmunity in the context of a permissive genetic background (Wagner, 2005, Imanguli et al., 2009). The process has been associated with a number of mechanisms including replacement of host antigen presenting cells (APCs) by donor APCs leading to indirect antigen presentation of alloantigens, persistence of alloreactive and autoreactive T lymphocytes, an imbalance of helper T cell type 1 (Th1) and helper T cell type 2 (Th2) responses, dysregulation of cytokine expression, and B lymphocyte auto- and allo-antibody production (Zhang et al., 2006, Socie et al., 2010). Collectively, these processes result in target tissue inflammation and injury that can progress to fibrosis and dysfunction (Ferrara et al., 2009).

There is great interest in identifying biomarkers for cGVHD risk prediction, diagnosis, and response assessment (Schultz et al., 2006, Paczesny et al., 2015). Oral lichen planus-like lesions are considered a diagnostic clinical feature of cGVHD and are readily accessible for

histopathological evaluation, however characterization of biologic markers remains limited (Mattsson et al., 1992, Sato et al., 2006, Jagasia et al., 2015). Immunophenotypic characterization, as an adjunct to clinical and histopathological findings, may provide a more in-depth understanding of the pathophysiologic mechanisms of cGVHD (Filipovich et al., 2005, Arai et al., 2011, Paczesny et al., 2015). The objective of this study was to characterize the immunohistopathologic features of oral mucosal cGVHD and the impact of intensive topical immunomodulatory therapy on the infiltrating cells.

Material and methods

Oral mucosal biopsies

Oral mucosa biopsy specimens were analyzed from patients with oral cGVHD enrolled in an open label phase II randomized trial (NCT00686855) at Dana-Farber/Brigham and Women's Cancer Center, Boston, USA (Treister et al., 2016). Adult (≥ 18 years old) with new-onset symptomatic oral cGVHD were recruited from the hematology/oncology clinics. Patients could not have been previously treated with intraoral topical corticosteroid and/or tacrolimus therapies, and systemic immunosuppressive regimens had to be stable for 4 weeks before study entry. The protocol was approved by the Institutional Review Board of the Dana-Farber/Harvard Cancer Center and all patients provided written informed consent in accordance with the Declaration of Helsinki. Oral mucosal cGVHD was defined based on the presence of hyperkeratotic lichenoid reticulations following the NIH consensus criteria for chronic GVHD (Filipovich et al., 2005).

The study group consisted of patients with new onset and treatment naïve oral cGVHD evaluated before (baseline) and one month after (end of treatment) intensive topical therapy (randomized to topical dexamethasone 0.1 mg/mL solution or tacrolimus 0.1 mg/mL solution, 5 minutes swish and spit, four times a day) who consented to have optional 4.0 mm punch biopsies collected at baseline and at the end of treatment visit. Biopsies were obtained under local anesthesia from a site demonstrating typical lichenoid reticular features, in most cases from the buccal mucosa or tongue dorsum, and placed in 10% phosphate-buffered formalin for routine paraffin section histology and immunohistochemical analysis.

In addition to the oral cGVHD tissue specimens, three control tissues were included in the immunohistochemical analysis. These consisted of clinically normal buccal mucosa or tongue obtained from autopsy (n = 5), archived specimens of oral acute GVHD (n = 3), and archived specimens of oral lichen planus (n = 5). Oral aGVHD was defined as the onset of oral mucosal erythema and ulcerations in the context of engraftment associated with the involvement of classical target organs (e.g. skin, liver, GI) (Ion et al., 2014). OLP was defined by the presence of bilateral reticular/lacey white lesions clinically, and histopathologically by a well-defined, band-like zone of lymphocytes, and signs of liquefaction degeneration based on the modified WHO diagnostic criteria (van der Meij & van der Waal, 2003).

Histopathology

Histologic features of oral cGVHD tissue specimens were qualitatively analyzed in 5.0µm sections by light microscopy for the presence of maturation disarray, basal cell layer squamatization and vacuolization, number and pattern of lymphocytes in the connective tissue and epithelium, and number of apoptotic cells, following the minimal histologic criteria for oral cGVHD based on the NIH consensus criteria (Shulman et al., 2015). The features were evaluated in 2–3 haematoxylin-eosin slides with 3–4 sections of each specimen, and the frequency of the histological features was determined for each sample.

Immunohistochemistry

Quantitative immunohistochemistry was performed to characterize the infiltrating mononuclear cells (CD3, CD4, CD8, CD20, CD103 and FoxP3), Langerhans cells (CD1a), mast cells (c-kit), macrophages (CD163) and endothelial cells (CD31 and CD62E) in the oral cGVHD specimens and controls. This panel was chosen based on previous data establishing the importance of these immune cells and pathways in cytotoxic injury to skin and squamous mucosa (Soares et al., 2005, Imanguli et al., 2009, Sato et al., 2006, Imanguli et al., 2014). In brief, 3.0µm serial sections were deparaffined, rehydrated, and processed for antigen retrieval. Sections were incubated with the primary antibodies (Table 2) at 4°C overnight, and then incubated with the secondary antibodies at room temperature for 60 minutes. The immunoreactivity was detected using NovaRed peroxidase or Vector Blue alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA, USA).

For double-labelled immunohistochemical staining (FoxP3⁺CD8⁺ and FoxP3⁺CD20⁺), sections were incubated with rabbit anti-human CD8 and mouse anti-human FOXP3 and then with peroxidase conjugated rabbit anti-goat antibody and alkaline phosphatase-conjugated horse anti-mouse antibody. Sections of human tissue were used as a positive control for all primary antibodies, as follows: spleen for CD3, CD4, CD8, CD20 and FoxP3; tonsil for CD1a, CD31, CD62E, CD163 and c-kit; and colon for CD103. Negative controls were obtained using a non-immune antibody of the same isotype and at the same concentration as the primary antibody. Immunoreactivity was designated positive when unequivocal nuclear, nuclear membrane or cytoplasmic labeling was detected. The positive cells were manually quantified in three randomly selected fields of 1mm² under high power (×40) magnification, and a median value was determined for each section as positive cells/mm². Sections/fields with folds, ruptures or strong background staining were not included in the analysis. The counting was carried out as follows: within the epithelium for CD1a; in the basal cell epithelial and subepithelial layers for CD3, CD4, CD8, CD20, CD103, CD163, and FoxP3; and in the subepithelial and deeper connective tissue area for c-kit. For the endothelial cell analyses, vessel density was calculated. In this case, each positive endothelial cell cluster of immunoreactivity (CD31 and CD62E) in contact with the field was counted as an individual vessel in addition to the morphologically identifiable vessels with a lumen, and a median value was determined for each section as number of vessels/mm².

Statistical analysis

Kruskal-Wallis or Mann-Whitney tests were used to determine significance in the difference of immunopositivity frequency distribution, as appropriate. The Wilcoxon signed-rank test was used to test for significance before and after four weeks of topical therapy. In all cases, the threshold for statistical significance was considered as a P-value <0.05. All statistical analyses were performed using GraphPad software (San Diego, CA, USA).

Results

Patient characteristics

Twelve patients with oral cGVHD who were randomized to receive either topical dexamethasone (n=8) or tacrolimus (n=4) consented to optional oral mucosal biopsies before and at the end of the study (Table 1). The median age was 55 (range 24–75) and the majority underwent reduced intensity conditioning, with all receiving peripheral blood stem cell grafts. Patients were enrolled at a median of 13 months post-allogeneic HCT (range 3–28), with the majority (9/12) being treated with systemic immunosuppressive therapy, which remained unaltered during the study period. At enrollment, all patients presented with prominent oral lichenoid changes, with variable involvement with erythema (n=11) and ulceration (n=7), with associated symptoms of oral sensitivity.

Histopathological features of oral mucosal cGVHD before topical therapy

Oral mucosal cGVHD biopsy specimens obtained at baseline were characterized by basal cell squamatization (12/12; 100%), sclerosis (11/12; 92%), apoptosis (9/12; 75%), lymphocytic exocytosis (8/12; 67%), and parakeratinization (8/12; 67%) (Figure 1a,b). In the lamina propria there was a lichenoid pattern of inflammation characterized by a band-like lymphocytic infiltrate at the interface between the basement membrane and the underlying connective tissue (11/12; 92%) (Figure 1b,2a). No cases exhibited generalized acute inflammation throughout the connective tissue, although infrequent foci of intraepithelial neutrophilic exocytosis were observed (3/12; 17%). Apoptotic cells were preferentially present in the basal epithelium, although occasional isolated apoptotic cells were also observed within the spinous and granular epithelial layers (Figure 1c). Multiple lymphocytes surrounding apoptotic cells (satellitosis) were also observed (Figure 1d).

Histopathological features of control tissues

Acute GVHD samples were characterized by extensive acute and chronic inflammation with apoptotic cells focally distributed at the interface area (3/3; 100%) (Figure 2g). OLP samples showed epithelial hyperplasia with hyperkeratosis and basal cell vacuolization, and a band-like lymphocytic infiltrate in the subjacent lamina propria (5/5; 100%) (Figure 2m). Normal buccal mucosa was characterized by nonkeratinized epithelium, broad and tapered rete ridges, and loose fibrovascular tissue in the lamina propria (3/3; 100%), and normal dorsal tongue was characterized by thick parakeratinized epithelium with filiform papillae (2/2; 100%); there was no acute or chronic inflammation noted (Figure 2s).

Characterization of infiltrating population cells of oral mucosal cGVHD specimens before topical therapy

Mononuclear cells (CD3, CD4, CD8, CD20, CD103 and FoxP3)—In oral cGVHD specimens, the infiltrating mononuclear cells primarily consisted of CD3⁺ and CD8⁺ T cells (Table 3). There was a similar number of CD3⁺ and CD8⁺ cells (59.6/mm² and 50.8/mm², respectively), both of which predominated over CD4⁺ cells (31.6 cells/mm²). There was moderate nuclear FoxP3 expression (28.9 cells/mm²) predominantly at the basement membrane interface following the same pattern of CD8⁺ immunoreactivity (Figure 2b). CD20⁺ cells were observed infrequently (0.41 cell/mm²). To confirm the immunophenotype of the FoxP3⁺ cell population, double immunoenzymatic labeling was performed and a very small population of FoxP3⁺CD8⁺ cells (2 cells/mm²), but no FoxP3⁺CD20⁺ cells were identified. In addition, CD103⁺ cells were observed at the basement membrane interface (31.3 cells/mm²) (Figures 2b,c,d).

Compared to the oral cGVHD findings, the same predominance of CD3⁺ and CD8⁺ cells over CD4⁺ cells was observed in aGVHD (CD3⁺ = 48.3 cells/mm²; CD8⁺ = 49 cells/mm²; CD4⁺ = 28.6 cells/mm²), OLP (CD3⁺ = 142.2 cells/mm²; CD8⁺ = 132.2 cells/mm²; CD4⁺ = 42.8 cells/mm²), and normal tissues (CD3⁺ = 13.6 cells/mm²; CD8⁺ = 8.6 cells/mm²; CD4⁺ = 5.4 cells/mm²), with the highest numbers of infiltrating cells in OLP specimens (Table 3). Intense FoxP3 expression (68.6 cells/mm²) was observed in OLP with a diffuse pattern and infiltrating the deep connective tissue compared to cGVHD, with low expression in aGVHD (8.3 cells/mm²) and almost no expression in normal tissues (0.8 cells/mm²). CD20⁺ cells were infrequent in aGVHD (1.6 cells/mm²), and normal tissues (0.2 cell/mm²), with a higher frequency in OLP (28.2 cells/mm²). The double labeling in control tissues demonstrated a small population of FoxP3⁺CD8⁺ cells in aGVHD (1 cell/mm²) and OLP (4 cells/mm²), and none in normal tissues (Figures 2h,n,t); there were no FoxP3⁺CD20⁺ cells identified in any of the control tissues. CD103⁺ cells were also observed in control tissues, with higher numbers in OLP (34.8 cells/mm²) compared with aGVHD (6.3 cells/mm²) and normal tissues (6.2 cells/mm²) (Figures 2j,p,v) (Table 3).

Characterization of CD8/FoxP3 and CD4/FoxP3 ratios

Overall there was a higher number of CD8⁺ cells than FoxP3⁺ cells in cGVHD (Figure 2b), with the ratio of CD8⁺ cells to FoxP3⁺ cells 3:1 for cGVHD, 5:1 for aGVHD, 2:1 for OLP, and 8:1 for normal tissues. There was also a higher number of CD4⁺ cells than FoxP3⁺ cells, with the CD4/FoxP3 ratio higher in cGVHD (2:1) and aGVHD (6:1) compared to OLP (1:1).

Characterization of mast cells (c-kit)

Expression of c-kit was observed in oral cGVHD in both the subepithelial infiltrate (3.2 cells/mm²) and in the deeper connective tissue (4.6 cells/mm²) (Figure 2e). Compared to oral cGVHD, control tissue c-kit expression within the subepithelial infiltrate and deeper connective tissue was greater in OLP (7.2 and 9.6 cells/mm²; $P=0.006$ and $P=0.008$), with no differences related to normal tissues (2.6 and 2.6 cells/mm²; $P>0.05$) and in aGVHD (2.0 and 2.6 cells/mm²; $P>0.05$) (Figures 2k,q,w) (Table 3).

Characterization of Langerhans cells (CD1a), macrophages (CD163), and endothelial cells (CD31 and CD62E)

CD1a⁺ cells were detected in the epithelium of oral cGVHD (2.4 cells/mm²), with scarce positive cells observed in the connective tissue (data not shown). There were no statistically significant differences in the number of CD1a⁺ cells between oral cGVHD and controls (oral aGVHD = 0.7 cells/mm²; OLP = 1 cell/mm²; normal tissue = 2.4 cells/mm²). There was a statistically significant increase in expression of CD163⁺ macrophages in oral cGVHD compared to normal tissues (31.3 cells/mm² and 12.1 cells/mm²; $P=0.007$), and in OLP compared to normal tissues (31.6 cells/mm² and 12.1 cells/mm²; $P=0.02$), but not in oral aGVHD (28 cells/mm²) (Table 3).

To assess vessel morphology and activation, CD31 and CD62E expression was assessed. Expression of both antibodies was observed at the endothelial cell junctions of oral cGVHD and control samples, with no statistically significant differences in the number of CD31⁺ and CD62E⁺ vessels between the groups (Table 3).

Impact of topical immunomodulatory therapy on immunohistopathological features of oral mucosal cGVHD

Topical therapy with dexamethasone or tacrolimus solutions resulted in morphologic changes in epithelial and connective tissues compared with pretreatment samples (Figure 3a, b, e, f, i, j, m, n). Three cases initially exhibited acute inflammation of the epithelium characterized by neutrophilic exocytosis; two of these completely resolved following treatment with dexamethasone (with one accompanied by clinical response, defined as 3-point reduction of sensitivity score from baseline to end of treatment), and the third showed neither histopathological changes nor clinical response following treatment with tacrolimus. In three of the twelve cases, basal cell squamatization completely resolved following treatment with dexamethasone ($n = 2$) and tacrolimus ($n = 1$), with associated clinical responses. The remaining nine cases demonstrated no changes in histopathological features from baseline to end of treatment, although five cases treated with dexamethasone had clinical responses. Lichenoid interface mucositis, noted in 11 cases prior to topical treatment, completely resolved in two cases treated with tacrolimus, with one demonstrating clinical response. The remaining nine cases showed no changes in histopathological features following topical treatment, but six cases experienced clinical response following treatment with dexamethasone.

Topical dexamethasone therapy (8 paired samples) resulted in a decreased number of CD4⁺ (from 35.4 cells/mm² to 24.2 cells/mm²; $P=0.04$) and CD103⁺ cells (from 30.8 cells/mm² to 13.2 cells/mm²; $P=0.02$) (Figure 3c,d,g,h). The expression of CD1a, CD3, CD8, CD20, CD31, CD62E, CD163, c-kit and FoxP3 was not significantly affected.

Topical tacrolimus therapy (4 paired samples) did not result in changes in the expression of the CD1a, CD3, CD4, CD8, CD20, CD31, CD62E, CD103, CD163, c-kit and FoxP3 (Figure 3k,l,o,p).

Discussion

In this study we characterized the immunohistopathological features in oral mucosal cGVHD, and evaluated the impact of topical immunomodulatory treatment on the histological and immunophenotypic features. aGVHD, OLP and normal oral mucosa specimens were used as controls for cGVHD for the immunohistopathological analysis. Although distinct, the three conditions (cGVHD, aGVHD and OLP) have similarities with respect to the mechanisms of tissue damage, including persistence of T cells and induction of apoptosis (Gilliam et al., 1996, Imanguli et al., 2008, Gupta & Jawanda, 2015). Most of the cGVHD cases exhibited lymphocytic exocytosis, apoptosis, and lichenoid interface mucositis prior to topical treatment, features that fulfilled the minimal criteria for the histopathological diagnosis of oral mucosal cGVHD (Demarosi et al., 2007, Shulman et al., 2015). Of note, we observed multiple lymphocytes surrounding apoptotic epithelial cells (i.e. satellitosis), which is considered the hallmark of target cell injury in aGVHD, and also described in cGVHD (Whitaker-Menezes et al., 2003, Murphy & Korngold, 2004, Imanguli et al., 2009).

Concerning the immunophenotypic characterization, we demonstrated that the infiltrating cells in oral mucosal cGVHD mainly consisted of CD3⁺ and CD8⁺ T cells, with a pattern of distribution at the interface area in the subepithelial connective tissue. Our data confirms previous studies demonstrating that oral cGVHD is characterized by a high number of CD3⁺ cells (Mattsson et al., 1992, Nakamura et al., 1996, Imanguli et al., 2009). The focal epidermotropic lymphocyte infiltrate confirms the important role of CD8⁺ cytotoxic T cell activity in the pathogenesis and progression of oral cGVHD (Fujii et al., 1988, Mattsson et al., 1992, Sato et al., 2006). In the context of effector cell trafficking, we investigated CD103, an important integrin that is preferentially expressed in intraepithelial lymphocytes, and binds to E-cadherin, which is expressed on the surface of squamous mucosa (Cepek et al., 1994, Andrew et al., 1996, Pribila et al., 2004, El-Asady et al., 2005). We found high expression of CD103⁺ cells in oral cGVHD. It is known that CD103 plays a key role in the alloimmune response to host epithelial tissues (i.e. intestinal epithelium) by CD8⁺ effector cells (Feng et al., 2002, El-Asady et al., 2005, Uss et al., 2006). Thus, it seems plausible that CD103⁺ cells may be involved in cGVHD-mediated oral mucosa injury through T cell homing to the interface area.

The FoxP3 transcription factor is considered the most reliable marker for regulatory T cells (Tregs), which suppress functions of T cells, and play a central role in regulating donor T cell-mediated alloreactivity (Corthay, 2009, Beres et al., 2011, Koreth & Ritz, 2013, Ligoeki & Niederkorn, 2015). In both aGVHD and cGVHD there is a reduced expression of circulating FoxP3⁺ cells (Miura et al., 2004, Zorn et al., 2005, Rezvani et al., 2006, Zorn et al., 2009). There are limited and conflicting studies characterizing FoxP3 expression in GVHD-affected tissue (Rieger et al., 2006, Wu et al., 2009), and few included oral mucosa (Imanguli et al., 2014). We found higher FoxP3 expression in cGVHD than in normal tissues, and OLP showed the highest number of FoxP3⁺ cells compared to the other groups (Figure 1 and Table 3). We substantiated immunophenotype of the FoxP3⁺ cell population as Tregs by double-labelled staining, and the relation between FoxP3⁺ cells and CD8⁺ and CD4⁺ cells in cGVHD sections showed lower numbers of FoxP3⁺ than CD8⁺ and CD4⁺

cells demonstrated by the CD8/FoxP3 and CD4/FoxP3 ratios (3:1 and 2:1, respectively). These findings suggest that although Tregs modulate the response of effector T cells, they failed to suppress the proliferation of CD8⁺ cytotoxic T cell activity in cGVHD and OLP at the tissue level.

Mast cells and macrophages are known for their role in the pathogenesis of GVHD through several immunological mechanisms, including secretion of pro- and anti-inflammatory cytokines, modulation of T cells response and the recruitment of effector T cells to the subepithelial inflammatory infiltrate (Murphy et al., 1994, Zhao et al., 1997, Zhao et al., 2001, Kolb, 2013, Shlomchik, 2007, Nishiwaki et al., 2009). In the present study, mast cells (c-kit⁺ cells) were found in oral mucosal cGVHD, as well as in the controls, and the major concentration of c-kit⁺ cells was observed at the deeper connective tissue compared to subepithelial infiltrate in all studied diseases, as previously reported (Zhao et al., 1998). Considering the known association between mast cells and sclerosis, it is worth relating the histologic sclerosis features found in most of oral cGVHD samples with the higher expression of c-kit in deeper connective tissue (Hugle, 2014). Regarding macrophages, we found increased number of CD163⁺ cells in aGVHD, cGVHD and OLP compared to normal tissues. Activation of CD8⁺ T cells results in increased secretion of cytokines that subsequently activates macrophages, thereby maintaining the inflammatory cascade (Reddy & Ferrara, 2003, Imaniguli et al., 2008). It is therefore not surprising the high expression of CD163⁺ cells in cGVHD tissues, as well as the aGVHD and OLP, given the high numbers of CD8⁺ T cells found in the inflammatory infiltrates.

The effectiveness of both topical corticosteroids and tacrolimus has been reported in oral cGVHD, as monotherapy or in combination (Eckardt et al., 2004, Mawardi et al., 2010, Noce et al., 2014). Dexamethasone is a corticosteroid that affects expression of transcription factor NF- κ B, suppressing the recruitment of cytotoxic T cells and reducing expression of multiple proinflammatory cytokines (Ge et al., 2012, Uva et al., 2012). Tacrolimus is a macrolide inhibitor of calcineurin which prevents T-cell activation and suppresses the release of various proinflammatory cytokines, including IL-4, IL-17, and IFN- γ (Tsuda et al., 2012). Our data demonstrates that topical therapy with dexamethasone or tacrolimus appears to reduce tissue inflammation within the epithelium or connective tissue mainly related to neutrophilic exocytosis and basal cell squamatization. Of the cases demonstrating lichenoid interface mucositis at baseline, most (9/11) showed no histopathologic changes suggestive of “response” or “improvement” following the topical treatment, although six cases experienced clinical response following treatment with dexamethasone. Additionally, topical treatment with dexamethasone reduced the number of CD4⁺ and CD103⁺ cells, supporting its direct immunosuppressive effect on T cells within the oral mucosa. The lack of correlation between histopathological changes and clinical response might be attributed to the small number of cases with biopsies before and after treatment (n=12). Although we believe that 1 month was a sufficient duration of therapy for both clinical response and immunohistopathological changes, some patients with oral mucosal cGVHD may require a longer duration of treatment and it is possible that histopathological changes would be observed at a later time, although this is entirely speculative.

In conclusion, we demonstrated that oral mucosal cGVHD is associated with increased numbers of CD3⁺, CD4⁺, CD8⁺, CD103⁺, CD163⁺ and FoxP3⁺ cells when compared with normal mucosa, confirming that oral cGVHD is largely T cell driven with participation of macrophages in maintaining the chronic inflammation. In addition, this study highlights differences in the immunophenotypic characterization of potential diagnostic and pathophysiologic significance between cGVHD and the other disorders studied (aGVHD and OLP), which indicates potential differences in the regulatory effects on the inflammatory response. This is the first study to demonstrate the impact of topical immunomodulatory therapy on immunohistopathological features of oral cGVHD, and our results provide new insight into potential mechanisms involved in response to topical therapy in oral mucosal cGVHD, with identification of specific mediators, including CD4⁺ and CD103⁺ cells, which may offer novel therapeutic targets. As oral cGVHD is easily monitored clinically and biopsies easily obtained, it provides a model target organ for study of cGVHD mechanisms and therapeutic response. Despite the study's limitations, our results suggest "trends" in expression of inflammatory cells that are preliminary and require validation in larger cohort studies.

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Figure 1.

Histopathologic features of oral cGVHD: (a,b) Buccal mucosa lesion showing epithelial hyperplasia and a band-like lymphocytic infiltrate at the mucosal-submucosal interface (Hematoxylin-eosin – H&E; original magnification at $\times 10$ and $\times 20$, respectively). (c) Apoptotic cells (arrows) and migration of lymphocytes into the lowermost epithelial layers (H&E; original magnification at $\times 40$). (d) Apoptotic cells (arrow) associated with lymphocytes known as satellitosis (H&E; original magnification at $\times 100$).

Figure 2. Histopathological features, and immunohistochemical (IHC) expression of CD3, CD4, CD8, FoxP3, CD103, and CD62E in oral mucosa specimens: (a–f) cGVHD, (g–l) aGVHD, (m–r) OLP and (s–x) normal tissues. (H&E: original magnification $\times 20$; IHC: original magnification $\times 40$).

Figure 3. Clinical and histopathological features, and immunohistochemical expression of CD4 and CD103 in oral cGVHD before and 1 month after topical therapy: (a–h) dexamethasone, or (i–p) tacrolimus (original magnification at $\times 40$).

Table 1

Characteristics of the oral cGVHD cohort.

N=12	N (%)
Age (median; range)	55 (24–75)
Sex	
Female	5 (42)
Male	7 (58)
Diagnosis	
AML	5 (42)
CLL	2 (17)
MDS/MPS	1 (8)
NHL	4 (33)
Stem cell source	
PBSC	12 (100)
Conditioning intensity	
Myeloablative	4 (33)
Non-myeloablative	8 (67)
Donor	
Matched related	6 (50)
Matched unrelated	6 (50)
Acute GVHD	
Any grade (I–IV)	7 (41)
Grade I	3 (18)
Grade II–IV	4 (23)
Systemic immunosuppressive therapy	
None	3 (25)
Tacrolimus	6 (50)
Sirolimus	6 (50)
Prednisone	3 (25)
NIH Oral cGVHD severity	
No symptoms	0 (0)
Mild	3 (18)
Moderate	8 (66)
Severe	1 (6)

AML = chronic myeloid leukemia; MDS = myelodysplastic syndrome; MPS = myeloproliferative syndrome; CLL = chronic lymphocytic leukemia; NHL = non-Hodgkin lymphoma; PBSC = peripheral blood stem cells; NIH = National Institute of Health

Table 2

Antibodies used for immunohistochemical staining.

Antibodies	Clone	Specificity	Source
CD1a	Monoclonal (7A7)	Langerhans cells	LifeSpan Biosciences, Seattle, WA, USA
CD3	Monoclonal (PS1)	Peripheral T cells	Abcam, Cambridge, MA, USA
CD4	Monoclonal (1F6)	Helper/inducer T cells	Invitrogen Corporation, Camarillo, CA, USA
CD8	Polyclonal	Cytotoxic T cells	Abcam, Cambridge, MA, USA
CD20	Monoclonal (L26)	B cells	DAKO, Carpinteria, CA, USA
CD31	Polyclonal	Endothelial cells	Bethyl Laboratories, Montgomery, TX, USA
CD62E	Monoclonal (16G4)	Endothelial cells	Neuromics, Edina, MN, USA
CD103	Monoclonal	Intraepithelial T cells	Abcam, Cambridge, MA, USA
CD163	Monoclonal (10D6)	Macrophages	Thermo Fisher Scientific, Waltham, MA, USA
c-kit	Monoclonal (5C8D4)	Mast cells	Novus Biologicals, Littleton, CO, USA
FoxP3	Monoclonal (259D)	Regulatory T cells	Biolegend, San Diego, CA, USA

Table 3

Comparative quantification of positive cells (mean $\pm$ SD of cells or vessels*/mm²) in cGVHD before topical therapy and control tissues.

Antibodies	Study groups				P-value						
	cGVHD (N = 12)	aGVHD (N = 3)	OLP (N = 5)	Normal oral mucosa (N = 5)	a	b	c	d	e	f	
CD1a	2.41 ± 2.50	0.66 ± 0.57	1 ± 1.2	2.4 ± 1.9	0.43	0.38	0.97	0.99	0.32	0.38	
CD3	59.67 ± 42.65	48.33 ± 46.2	142.20 ± 83.01	13.6 ± 10.11	0.44	0.08	0.004	0.08	0.16	0.007	
CD4	31.67 ± 10.9	28.6 ± 15.7	42.8 ± 24.2	5.4 ± 3.6	>0.99	0.62	0.001	0.39	0.03	0.007	
CD8	50.8 ± 22.92	49 ± 50.86	132.2 ± 68.88	8.6 ± 4.45	0.94	0.003	0.0003	0.14	0.57	0.007	
CD20	0.41 ± 0.66	1.66 ± 0.57	28.2 ± 24.24	0.2 ± 0.44	0.02	0.0002	0.70	0.03	0.03	0.007	
CD31*	11.17 ± 4.72	14.67 ± 5.77	8.8 ± 2.28	8.8 ± 0.83	0.26	0.39	0.45	0.21	0.35	0.99	
CD62E*	1.58 ± 1.50	3.66 ± 1.52	1.2 ± 1.09	1.6 ± 0.89	0.06	0.77	0.87	0.08	0.07	0.64	
CD103	29.5 ± 14.67	6.33 ± 4.04	34.8 ± 12.79	6.2 ± 3.76	0.008	0.39	0.0006	0.03	0.99	0.007	
CD163	31.33 ± 18.86	28 ± 4.35	31.6 ± 9.29	12.2 ± 8.3	>0.99	0.62	0.03	0.85	0.07	0.02	
c-kit											
<i>Subepithelial area</i>	3.25 ± 2.22	2 ± 1.73	7.2 ± 3.96	2.6 ± 1.94	0.32	0.02	0.61	0.45	0.83	0.03	
<i>Deeper connective area</i>	4.58 ± 3.20	2.66 ± 2.51	9.6 ± 4.09	2.6 ± 2.07	0.44	0.01	0.35	0.03	0.99	0.007	
FoxP3	28.92 ± 19.76	8.33 ± 7.09	68.6 ± 27.98	0.8 ± 0.83	0.10	0.01	0.0003	0.03	0.07	0.007	

^a cGVHD versus aGVHD;

^b cGVHD versus OLP;

^c cGVHD versus normal oral mucosa;

^d aGVHD versus OLP;

^e aGVHD versus normal oral mucosa;

^f OLP versus normal oral mucosa.