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Immunohistochemical Analysis of Lymphocyte Populations in Acute Skin Rejection: The University Health Network Addition to the Banff Classification

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Background: Acute rejection in vascularized composite allotransplantation has been identified using the Banff 2007 working classification. We propose an addition to this classification based on histological and immunological assessment within the skin and subcutaneous tissue.
Methods: Biopsies from vascularized composite transplant patients were obtained at scheduled visits and whenever skin changes occurred. Histology and immunohistochemistry were performed on all samples, looking at infiltrating cells.
Results: Observations were made specifically related to each component of the skin, including the epidermis, dermis, vessels, and subcutaneous tissue. Our findings led to the establishment of the University Health Network addition of skin rejection.
Conclusions: The high rate of rejection where the skin is involved requires novel techniques for early detection. The University Health Network skin rejection addition can serve as an adjunct to the Banff classification. *(Plast Reconstr Surg Glob Open 2023; 11:e4831; doi: 10.1097/GOX.0000000000004831; Published online 3 March 2023.)*

INTRODUCTION

Vascular composite allotransplantation (VCA) involves the transfer of fat, muscle, tendon, bone, cartilage, nerves, vessels, and skin to regions that cannot be salvaged with conventional surgical techniques.[1] Since the first hand transplantation in 1964, there has been a significant increase in the number of VCA performed.[2] Although VCA is not lifesaving, its purpose is to improve quality of life (ie, life-enhancing).[3] Particularly, its potential for tissue reconstruction after birth defects, trauma, or tumor resection cannot be understated.[4] However, the practicality and feasibility of its use has been limited because patients remain on lifelong immunosuppression, which makes them prone to lymphoproliferative disease, opportunistic infections, chronic kidney failure, and other complications.[5] Acute rejection remains a major concern in this population, as approximately 85% of patients experience at least one episode of acute rejection, and over 50% experience multiple episodes.[6,7] Skin rejection has been presumed to be primarily a T-cell-mediated immune response, similar to the mechanisms previously described in solid organ allograft rejection.[8]

Skin-containing allografts provide a unique opportunity compared with solid organ transplantation because the skin is easily accessible and can also be monitored for changes clinically.[9] The skin has been recognized as the main target of the immune response in acute rejection.[9] Cendales et al proposed a classification system for acute skin rejection of VCA following histological analysis of biopsies collected in human limb allografts and abdominal walls at varying stages of rejection.[10,11] The Banff scale is graded 0 to 4, where grade 0 shows nonspecific changes, no or rare inflammatory cells. Grade 1 represents mild rejection characterized by mild, superficial perivascular infiltrate with no infiltration of inflammatory cells into the epidermis, but a few lymphocytes seen infiltrating into the adnexal glands. Grade 2 represents moderate rejection and is characterized by an inflammatory infiltrate more intense than in grade 1 and predominantly perivascular. Infiltration of lymphocytes into the epidermis and

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the hair shaft is also observed, and very little keratinocyte necrosis is seen. Grade 3 represents severe rejection. In addition to prominent perivascular inflammation, there is a band-like infiltrate just beneath the dermal-epidermal junction. Infiltration of the epidermis and adnexal glands is also present. Grade 4 demonstrates a necrotizing acute rejection with frank necrosis of the epidermis or other skin structures. This Banff 2007 working classification of skin pathology is considered the gold standard. However, it does present with some challenges. It is based on histology alone, depends on dermatopathologist experience, and might not pick up on early signs of acute rejection. Early recognition of rejection is critical for optimal management and prevention of chronic rejection and VCA loss.[12]

The aim of this study was to describe an addition to the Banff classification based on histological and immunological assessment of epidermal and dermal markers within each layer of the skin. This will allow for early identification of acute rejection and can serve as an adjunct to the Banff classification.

Takeaways

Question: Acute rejection in vascularized composite allotransplantation is difficult to diagnose. The high rate of rejection in vascularized composite allotransplantation where the skin is involved requires novel techniques for early detection.

Findings: Biopsies from vascularized composite allotransplants have yielded histologic observations related to each component of the skin, including the epidermis, dermis, vessels, adnexal structures, and subcutaneous tissue. Our findings led to the establishment of the University Health Network addition to the Banff classification of skin rejection.

Meaning: The University Health Network addition can serve as an adjunct to the Banff classification of rejection.

MATERIALS AND METHODS

Specimens

This study was approved by the research ethics board at the Toronto General Hospital Research Institute, University Health Network (Toronto, ON, Canada) and was performed in compliance with relevant guidelines. Informed consent was obtained from all patients. Typical immunosuppression regimen includes steroids, cyclosporine (Cyclosporin, Novartis, USA) and/or azathioprine (Imuran, Prometheus Laboratories Inc, USA). Specimens were obtained from one upper extremity transplant (below elbow) and one patient who underwent solid organ transplantation and a sentinel flap. Procured specimens included multiple samples of skin and subcutaneous tissue obtained by 3-mm punch biopsies. Local anesthetic was provided (1% xylocaine with 1:200,000 epinephrine) before chlorohexidine prep of area of biopsy. At least three samples were collected from different areas on the extremity transplant and the sentinel flap at each time point. Regular timepoints were the following: 1 week, 2 weeks, 1 month, 2 months, 3 months, 6 months, 9 months, 12 months, 1 year, 2 years, and 3 years posttransplantation. Additional samples were also collected whenever skin changes were observed, such as a rash or discoloration. Collected specimens included up to 3 years following transplantation. Samples were graded by a trained dermatopathologist. Episodes of acute rejection were treated with steroids and possible increase in baseline medication.

Histology and Immunohistochemistry

Skin and subcutaneous tissue biopsies were fixed in 10% phosphate-buffered formalin for 24 hours and paraffin embedded. The samples were sectioned in 5-µm-thick slides for hematoxylin and eosin (H&E) and immunohistochemical staining. Specifically, the paraffin samples were cleared and rehydrated through a series of xylene and ethanol and stained with hematoxylin (Sigma-Aldrich, St. Louis, Mo.) and eosin (Thermo Fisher Scientific, Cheshire, UK). Presence of CD3, CD4, CD8, and CD20 was evaluated using VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, Calif.) for anti-human CD3 (1:1000, Agilent), anti-human CD4 (1:200, Agilent), anti-human CD8 (1:500, Abcam) and anti-human CD20 (1:500, Agilent). Briefly, proteinase K (Dako, Carpinteria, Calif.) was utilized for antigen retrieval and nonspecific binding was blocked using normal horse serum. For isotype control, mouse IgG was utilized at the same dilution as that of the primary antibody. Slides were incubated with biotinylated secondary antibody for 30 minutes followed by 30-minute incubation with RTU VECTASTATIN ABC Reagent. Then 30 µl of ImmPACT DAB Reagent was diluted in 1 mL of ImmPACT DAB Dilutent (Vector Laboratories, Burlingame, Calif.) and applied to the slides. They were counterstained with Harris Hematoxylin solution (Sigma-Aldrich, St. Louis, Mo.) and dehydrated and cleared in ethanol and xylene, respectively.

RESULTS

Patients included in this study showed signs of rejection ranging from swelling to rash and/or asymptomatic erythematous scaly papules that were limited to the allograft. Treatment involved systemic or topical steroids and increased baseline immunosuppression. Biopsies were performed at scheduled visits and if any skin changes occurred. These biopsies demonstrated Banff grade ranging from 0 to 3. None of our transplant patients showed signs of grade 4 rejection.

We observed Banff grade 1 rejection characterized by mild, superficial perivascular infiltrate with no infiltration of inflammatory cells into the epidermis, but a few lymphocytes were seen infiltrating into the adnexal glands. We specifically focused on each of the layers of the skin (Fig. 1) and found signs of spongiosis within the epithelium (Fig. 1i), perivascular lymphocytic infiltration within the dermis (Fig. 1ii) with minimal signs of inflammation around the adnexal structures and the subcutaneous tissue (Fig 1iii).

Banff grade 2 represented moderate rejection and was characterized by an inflammatory infiltrate more intense than in grade 1 and predominantly perivascular. Infiltration of lymphocytes into the dermis and the hair shaft was also observed, and very little keratinocyte necrosis was seen. Our results (Fig. 2) showed changes in the epidermis consistent with exocytosis. The dermis showed perivascular and eosinophilic infiltrate (Fig. 2i) with early signs of vasculopathic changes and vasculitis (Fig. 2ii). The adnexal structures, including the hair follicles and the sweat glands, demonstrated moderate inflammation, whereas the subcutaneous tissue showed early vasculopathic changes and vasculitis (Fig. 2ii, iii).

Banff grade 3 represented severe rejection. In addition to prominent perivascular inflammation, there was a band-like infiltrate just beneath the dermal-epidermal junction. Infiltration of the dermis and adnexal glands was also present. We have observed within the epidermis, signs of interface change (Fig. 3i). Vasculitis and perineural inflammation were present within the dermis (Fig. 3i, ii). The adnexal structures showed severe inflammation with vasculitis affecting the subcutaneous tissue (Fig. 3iii). Banff grade 4 demonstrated a necrotizing acute rejection with frank necrosis of the epidermis or other skin structures. Although none of our patients showed Banff grade 4, we would expect signs of

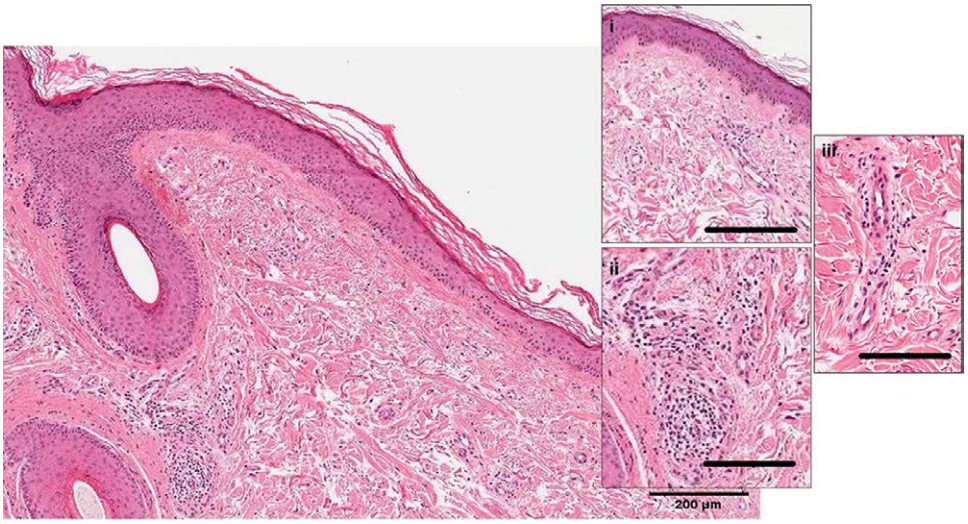

Fig. 1. Histology on skin biopsies. H&E staining corresponding to Banff grade 1 rejection (A). No infiltration of lymphocytes is seen at the level of the dermis, yet signs of spongiosis are present (i). Perivascular mononuclear cells are surrounding the adnexal glands (ii) and vessels (iii). Scale bar = 200 µm. Scale bar in box is 100 µm.

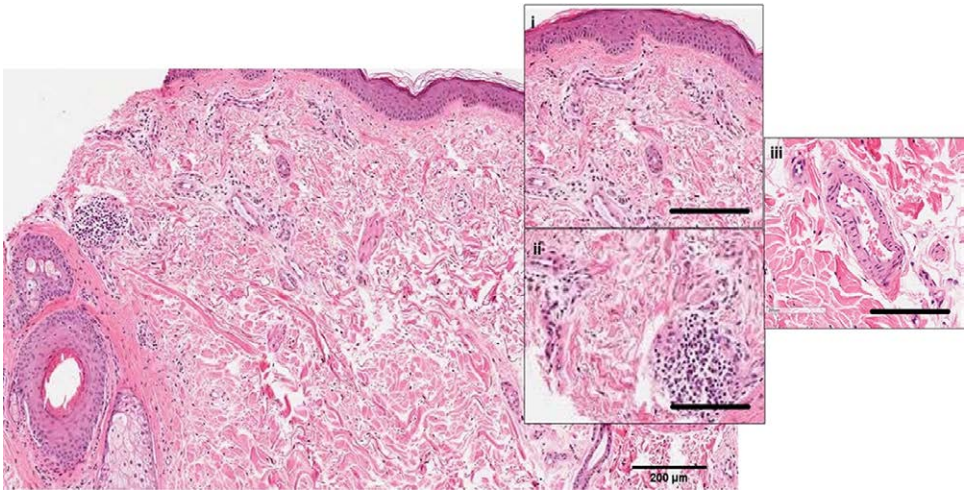

Fig. 2. Histology on skin biopsies. H&E staining corresponding to Banff grade 2 rejection (A). The changes within the epidermis are consistent with exocytosis (i), whereas the dermis shows lymphocytic perivascular and eosinophilic infiltrates (ii) and vasculitis (iii). Scale bar = 200 µm. Scale bar in box is 100 µm.

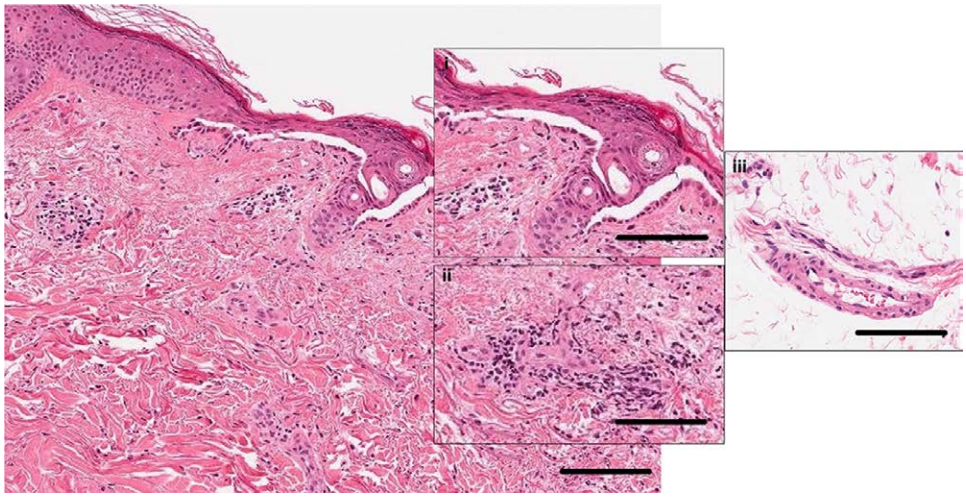

Fig. 3. Histology on skin biopsies. H&E staining corresponding to Banff grade 3 rejection. Signs of interface change are seen within the epidermis (i) with inflammation around the adnexal structures (ii) and nerves and vessels (iii). Scale bar = 200 μm. Scale bar in box is 100 μm.

necrosis affecting the adnexal structures and subcutaneous tissue.

Immunohistochemistry was performed, looking at lymphocyte populations at Banff grade of classification. Both CD4+ and CD8+ lymphocyte populations were identified in grade 2 and grade 3 rejection with the largest populations being CD4+ (Fig. 4). Interestingly, these cells seem to be centered around the vessels and adnexal structures (Fig. 4).

Our findings have led to the development of the University Health Network addition for skin rejection (Fig. 5). This can be used as an adjunct to the Banff grade

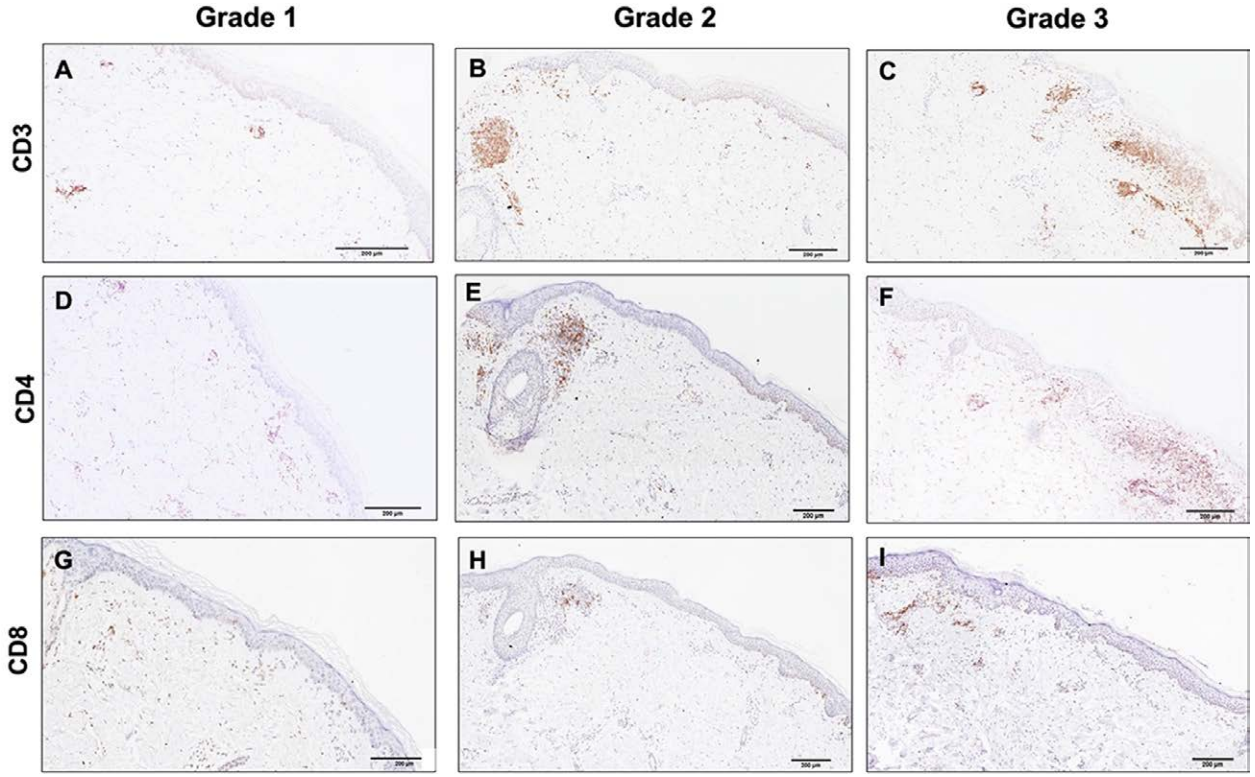

Fig. 4. Immunohistochemistry of skin samples looking at CD3+, CD4+, and CD8+ cells. Grade 2 and 3 rejection show a high infiltration in the subdermal regions and around the vessels and adnexa. Scale bar = 200 μm.

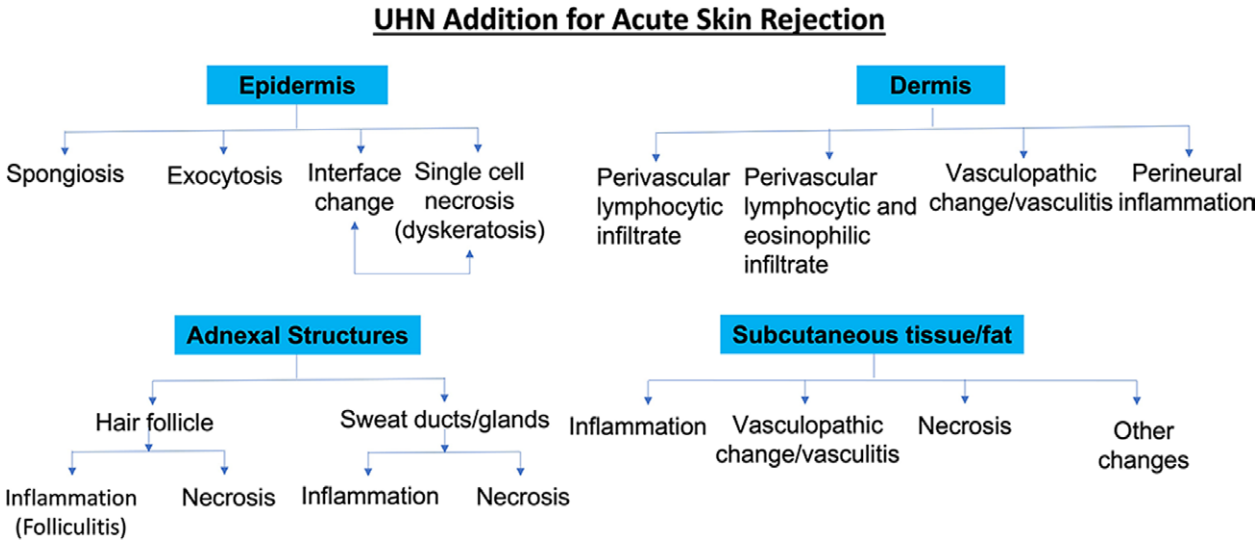

Fig. 5. University Health Network addition for acute skin rejection showing expected changes at level of epidermis, dermis, adnexal structures, and subcutaneous tissue/fat.

and focuses on different components within the skin and soft tissue. Particularly, the changes within the epidermis ranged from spongiosis, exocytosis, interface change, and necrosis. The changes within the dermis were reflected by a perivascular lymphocytic infiltrate, a perivascular lymphocytic and eosinophilic infiltrate, vasculopathic change/vasculitis, and perineural inflammation. Changes related to the hair follicles and sweat duct glands included changes from inflammation to necrosis. The subcutaneous tissue/fat showed inflammation, vasculopathic change/vasculitis, and necrosis.

DISCUSSION

Acute rejection in vascularized composite allotransplantation can be seen clinically with changes such as erythema, edema, dermatitis, and eventual necrosis. Despite these changes, the clinical appearance of the skin has no definitive predictive value in isolation, and histologic studies are absolutely required not only to establish the severity of rejection, but also to discriminate between acute rejection and other infectious, allergic, autoimmune, and dermatitic skin diseases.[13]

The monitoring of acute skin rejection within VCA has typically been performed using a biopsy of the skin and subcutaneous tissue and interpreted using the Banff 2007 working classification of skin pathology.[10,11] This scale is graded 0 to 4 and represents mild, moderate, and severe rejection, and necrosis. It is characterized by superficial perivascular infiltrate with or without infiltration of inflammatory cells and lymphocytes into the adnexal glands. The Banff classification system relies almost exclusively on histopathology. Its strengths are the international uniformity in reporting allograft pathology. This standardized grading system has provided objectivity for publication, data sharing, and statistical analysis, all of which are vital for patient management, clinical trials, and research; however, limitations do exist.[14] Inherent shortcomings stem from the Banff classification's almost exclusive reliance on histopathological characterization of rejection. Thus, important limitations exist in differentiating rejection from other T-cell-dominated inflammatory skin conditions as well as inadequate intra- and interobserver reproducibility.[14]

The latter deficiency in the Banff classification is most pronounced at the interface between borderline acute rejection and chronic rejection, which is where precision is needed the most.[15] An analogous situation is present in VCA with reports of mild histopathological signs of rejection with complete absence of clinical signs of rejection. This variability appears to be particularly noted in the differentiation between grade 1 and grade 2 rejection.[1] In the current 2007 Banff schema, the major difference between these two categories is between "mild perivascular inflammation and "moderate perivascular inflammation."[1] However, the terms "mild" and "moderate" are not defined by an objective set of parameters.

Although the Banff classification provides a helpful approach for evaluating acute rejection, new histologic patterns observed from years of VCA have not been encompassed in the 2007 criteria. These include dermal sclerosis encasing the dermal capillaries, sweat gland atrophy, lichenoid changes.[16–18] and capillary thrombosis in the upper dermis, with a dense perivascular infiltrate[19] which are thought to be signs of chronic rejection. Etra et al proposed a VCA skin rejection classification in swine which they called the "modified Banff criteria."[20] This focused only on infiltrating inflammatory cells leading to dermal inflammation and largely epidermal involvement focusing on inflammation followed by necrosis. Lian et al described pathologic findings during episodes of rejection which uniformly consisted of perivascular lymphoid infiltrates; cell sloughing into vessel lumen, termed lymphoid vasculitis; lymphocyte migration into the epidermis; and pilosebaceous structures and epithelial apoptosis spatially

associated with infiltrating lymphocytes.[21] They also describe various degrees of lymphocytic vasculitis around the superficial venules, lymphocytes around the epidermis with eventual spongiosis, and thickening followed by apoptosis.[21] Rosales et al proposed a new classification looking at perivascular cells within the dermis, perivascular dermal infiltrate, epidermal infiltrate, transepidermal infiltrate, vessel endarteritis, keratinocyte apoptosis and necrosis, chronic allograft vasculopathy, and capillaritis.[22]

Given the heterogeneity of skin-containing allografts, it is important to look for other histologic features and to organize all histologic findings. We propose the use of the University Health Network Classification of rejection as an adjunctive tool to the Banff grade. In this addition, we focused on each component within the skin. More importantly, we looked at changes within the epidermis that are not limited to lymphocytic infiltration. As shown in our immunohistochemistry, lymphocytic proliferation was a classic feature within the dermis, and findings such as spongiosis, exocytosis, and interface change can be identified and represent signs of acute rejection.

The high rate of rejection in VCA where skin is involved requires novel techniques for early detection. The Banff grade focuses particularly on the degree of lymphocytic infiltration and might not allow for early detection when changes are more subtle. Although systematic and prospective examination of larger cohorts of patients is required to further validate this tool and to create grades of classification, this scale has the potential of being used as an adjunct where an experienced pathologist can look at different structures of the skin and connotate these differences, leading to better outcomes. Predictive tools in plastic surgery play a very important role in establishing outcomes and prognosis.[23,24] Although this study has its limitations in that the predictive ability needs to be further validated, it can play a bigger role when combined with other parameters, including clinical and histological assessments of rejection. Further analysis and correlation with multiple centers, particularly looking at infiltrating lymphocytes, will be required.[25,26]

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