OPEN

A Skin Rejection Grading System for Vascularized Composite Allotransplantation in a Preclinical Large Animal Model

Joanna W. Etra, MD,[1,2] Michael J. Grzelak, BS,[2,3] Samuel A.J. Fidder, MD,[2] Keli Kolegraff, MD, PhD,[2] Steven Bonawitz, MD,[4] Jaimie Shores, MD,[2] Byoungchol Oh, DVM, PhD,[2] Damon S. Cooney, MD, PhD,[2] Sarah E. Beck, DVM, PhD, DACVP,[5] and Gerald Brandacher, MD[2]

Background. The Banff Criteria have been accepted as a system for grading histological rejection in graft skin in human vascularized composite allotransplantation (VCA). Preclinical swine hindlimb transplantation models have an important role in translational studies in VCA. However, unified grading criteria for rejection in swine skin have not yet been established. **Methods.** Two hundred fourteen swine skin biopsy specimens were reviewed, including 88 native skin biopsies and 126 specimens from the skin component of heterotopic swine hindlimb transplants. Thorough review was performed in a blinded fashion by an expert veterinary pathologist with attention paid to the applicability of the Banff criteria as well as specific histologic characteristics and trends. Clinical and histopathologic rejection scores were then directly compared. **Results.** Two hundred fourteen specimens reviewed showed significant similarities between swine and human skin, as previously published. Notable swine-specific characteristics, including paucicellular infiltration with rare epidermal cell infiltration or necrosis, were accounted for in a proposed grading system that parallels the Banff Criteria. **Conclusions.** This comprehensive grading system, based on the Banff Classification for skin rejection in VCA, provides a standardized system for more accurate comparison of rejection in preclinical swine VCA models.

(*Transplantation* 2019;103:1385–1391)

INTRODUCTION

Vascularized composite allotransplantation (VCA) is an increasingly utilized reconstructive procedure for patients with upper extremity amputation or devastating facial tissue defects. Although the skin component has been considered an obstacle for widespread application of VCA due to its high antigenicity and thus requiring the use of high-dose multidrug maintenance immunosuppression,[1-3] it also offers a unique opportunity for rejection monitoring as clinical visualization and biopsy collection are considerably more facile than in solid organ transplantation.[4] As of now, along with clinical assessment of the graft, biopsy and histologic evaluation of the skin component is the gold standard in monitoring for episodes of acute rejection.[5-8] Thus, the ability to grade rejection histologically

Received 19 December 2018. Revision received 13 February 2019.

Accepted 14 February 2019.

[1] Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD.

[2] Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory, Johns Hopkins University School of Medicine, Baltimore, MD.

[3] Johns Hopkins University School of Medicine, Baltimore, MD.

[4] Department of Surgery, Cooper Medical School of Rowan University, Camden, NJ.

[5] Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, MD.

J.W.E. participated in research design, performed specimen collection and processing, did the data analysis, and wrote the article. M.J.G. participated in research design, performed specimen collection and processing, assisted with review and specimen analysis, and assisted with article preparation. S.A.J.F. performed specimen collection and processing, assisted with review and specimen analysis, and assisted with article preparation. K.K. participated in research design. S.B. performed the surgical procedures for the transplanted grafts, participated in sample generation. J.S. performed the surgical procedures for the transplanted grafts, participated in sample generation. B.O. performed the surgical procedures for the graft retrieval, assisted with animal care and monitoring, and assisted with specimen collection and processing. D.S.C. assisted with study design, performed the surgical procedures for the transplanted grafts, and participated in sample generation. S.E.B. participated in research design, did the primary analysis and specimen review, and assisted with manuscript preparation. G.B. participated in research design and general consultation and oversight to the project..

The authors declare no conflicts of interest.

J.W.E., M.J.G., S.E.B., and G.B. contributed equally to this article.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

Correspondence: Sarah E. Beck, DVM, PhD, DACVP, Johns Hopkins University School of Medicine, Miller Research Building, Suite 833, 733 N. Broadway St, Baltimore, MD 21205. (sbeck8@jhmi.edu).

ISSN: 0041-1337/19/10307-1385

DOI: 10.1097/TP.0000000000002695

<zdoi;10.1097/TP.0000000000002695>

is of great importance in VCA treatment, monitoring, and maintenance. For VCA patients, the Banff 2007 Working Classification was formalized to make uniform the pathologic grading of rejection in skin biopsies.[7] This system provides a structure and guideline to human skin pathologic diagnosis. Based on a grade of 0 to 4, these criteria outline the histopathologic findings through different stages of rejection, as summarized in Table 1.

As VCA is a relatively young field with few human patients and studies, preclinical and translational models are especially important in evaluating outcomes and improvements in treatment regimens as well as immunological monitoring.[9] It is well established that swine skin is comparable to human skin in clinical and histopathological settings.[10-18] Anatomically, both pig and human skin have similar thickness ratios of dermis to epidermis, density of hair follicles, pigmentation (breed dependent), and dermal connective tissue composition.[18] Pig skin, like human skin, is also tightly adherent to the subcutaneous layer, in contrast to rodent skin.[18] Furthermore, pigs are easy to work with as they are easily trained to human contact, and their large size, which could be an obstacle in housing and care, can be mitigated through the use of minipig breeds rather than standard-sized breeds. Specifically, the swine hindlimb allotransplantation model is a well-described large-animal model that can be used to adequately assess the immunologic aspects of VCA as comparable to human allografts.[19] Despite common use of swine for VCA research, there is a need for more detailed histopathologic characterization of the unique characteristics of VCA rejection in the skin of minipigs compared to humans.[20] Given the importance of an analogous model, it is vital that we accurately and reproducibly classify histologic findings in skin samples from swine VCA. Thus, we present here a modified grading system, based on the Banff Classification, for acute skin rejection in VCA in a preclinical swine model.

MATERIALS AND METHODS

Study Cohort

All studies were performed with approval from the Johns Hopkins University Institutional Animal Care and Use Committee (IACUC). Hindlimb transplants were performed as previously described by our group[19] across full and partial swine leukocyte anitigen-mismatched Massachusetts General Hospital (MGH) minipigs from 2011 to 2018 under multiple different study protocols. One hundred thirty-seven animals were evaluated for inclusion into the study, which is, to our knowledge, the largest cohort of VCA-model minipigs reported. Biopsies included in the review were those with episodes of rejection with concurrent biopsy and clinical photograph available. Control specimens evaluated were native skin samples and ischemic skin without rejection (spontaneous vascular thrombosis in the first postoperative week with subsequent ischemic graft failure). This ischemia can be differentiated from rejection clinically, as they have notably different natural history. Animals that we allow to reject immediately postoperatively (no treatment) follow a reproducible pattern of severe edema with erythema, significant graft warmth, purple discoloration of the graft increasing in hue starting postoperative day 4 or 5, and subsequent bullae formation with epidermal sloughing. The grafts lost due to ischemia all had immediate pallor and cool temperature (both on clinical exam and infrared thermography), light blue discoloration of the graft with moderate edema beginning around postoperative day 5, and subsequent blackening/necrosis of the graft to full eschar. Examination of graft vasculature for patency was performed at animal euthanasia. Biopsies were evaluated at different stages of clinical rejection, and the treatment and timing of biopsies were specific to the different studies into which the animals were enrolled.

TABLE 1.

The Banff 2007 working classification of skin-containing composite tissue allograft pathology[7]

Grade	Findings
Grade 0	No or rare inflammatory infiltrates
Grade 1	Mild; mild perivascular infiltration; no involvement of the overlying epidermis
Grade 2	Moderate; moderate-to-severe perivascular inflammation with or without mild epidermal and/or adnexal involvement (limited to spongiosis and exocytosis); no epidermal dyskeratosis or apoptosis
Grade 3	Severe; dense inflammation and epidermal involvement with epithelial apoptosis, dyskeratosis, and/or keratinolysis
Grade 4	Necrotizing acute rejection; Frank necrosis of epidermis or other skin structures

Sample Preparation

Cutaneous biopsies were obtained from either the skin paddle of a heterotopic hindlimb transplant of a swine or native animal skin. Procedures were performed using a 5-mm punch biopsy. Specimens were immediately fixed in formalin for a minimum of 24 hours. The more recent samples were transitioned into ethanol after 24 hours, but the initial samples in the cohort remained in formalin until embedding. All of the specimens were embedded in paraffin and then stained using a standard hematoxylin and eosin staining protocol. Immunohistochemical analysis of the samples was performed to identify global trends in infiltrating cellular phenotype. Specimens were evaluated for the presence of T cells, B cells, regulatory T cells, and macrophages (CD3: Dako A0452; CD20: Biocare ACR3004B; FoxP3: eBioscience 14-5773-82; AntiS100A9: Thermo MA1-80446). Neutrophils and eosinophils were distinguished by morphologic appearance on hematoxylin and eosin.

Clinical Grading Criteria

Clinical rejection scores of the VCA allografts were assigned based on the clinical features of the graft skin (Figure 1): Grade 0 shows no difference between graft skin and native skin; Grade 1 has mild erythema; Grade 2 has moderate erythema with the beginning of scaling and scabbing; Grade 3 has severe erythema and scabbing with areas of epidermolysis; and Grade 4 has full-thickness graft epidermolysis with areas of necrosis.

Histopathologic Grading Criteria

Clinical rejection grading was given at timepoints corresponding to each biopsy, based on the review of prior clinical assessment and photodocumentation. All graft skin biopsies were reviewed retrospectively in a blinded fashion by a board-certified veterinary pathologist (S.E.B.) and

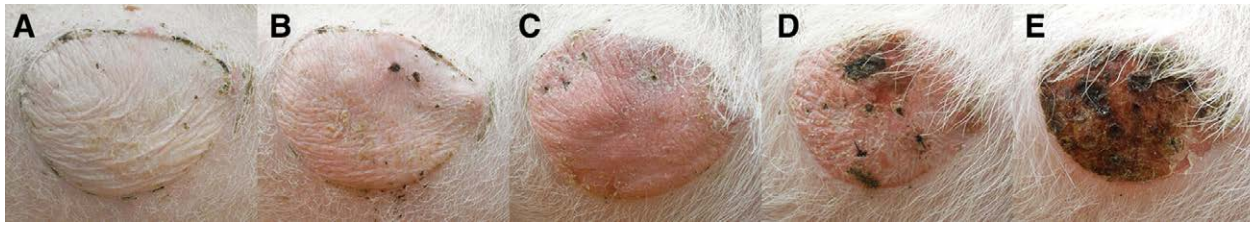

FIGURE 1. Examples of each clinical rejection grade in a swine hindlimb transplant performed in a full SLA-mismatch. Grade 0 (A) shows no difference between graft skin and native skin; Grade 1 (B) has mild erythema; Grade 2 (C) has moderate erythema with mild scaling and scabbing; Grade 3 (D) has severe erythema and scabbing with areas of epidermal sloughing; and Grade 4 (E) has full graft epidermolysis and necrosis.

assigned a rejection score (Table 2). This rejection score takes into account both the amount of dermal inflammation (Figure 2) and the presence of epidermal inflammatory infiltration and/or necrosis (Figure 3). The full grading system is described in detail in the Results section.

Statistical Analysis

Data were collected and maintained in a database created using Microsoft Excel (v 16.16.2). All categorical variables were described as count (percent). Statistical analyses were performed using Stata/IC 15.1 (StataCorp LLC). A mid-p McNemar test was utilized for data analysis.

RESULTS

Of the swine skin samples included in this study, a cohort of 214 samples were evaluated in a blinded fashion by a board-certified veterinary pathologist with extensive experience with swine histology. The cohort included samples of VCA graft skin over multiple timepoints and treatment regimens, ranging from posttransplant day 0 to posttransplant day 509. Within this group of tissue samples, 88 were native skin biopsies taken at the same time as the samples biopsied from graft skin. The cohort also included 6 samples from ischemic controls to account for differences in nonrejection inflammatory states (Table 3). The clinical rejection scores, based on the presence of severe erythema, scaling/scabbing, epidermolysis, or necrosis (Figure 1), were assigned to the graft at the timepoint the biopsy was taken. Out of the graft biopsies with associated available photograph on the corresponding day (n = 126), 37 were assigned clinical Grade 0 rejection, 54 assigned Grade 1, 16 Grade 2, 6 Grade 3, and 13 Grade 4 (Table 4).

The pathologist assessing each of these samples assigned, in a blinded fashion, a grade of histologic rejection to the sample, based on the Banff grading system with attention to the degree of inflammation present. To score inflammation, the number of dermal lymphocytic perivascular cuffs was averaged over at least three 20× fields. Perivascular cuffs were defined as circumferential inflammatory cells immediately surrounding a blood vessel. If perivascular cuffs were present in the sample, the cuff thickness was estimated based on the number of lymphocytes from the blood vessel to the outer edge, for which the number was also averaged over at least three 20× fields. As perivascular cuffs are often not completely symmetrical in nature, the thickest portion of the cuff was used to define the degree of inflammation present (Table 5). Samples were then given an overall inflammation score, based on the following criteria: "none" = no perivascular cuffs; "minimal" = <5 cuffs, no more than 2 cells thick; "mild" = <5 cuffs, 3 cells thick or more; "moderate" = 5 to 15 cuffs, any thickness; "severe" = >15 cuffs, any thickness (Figure 2). Swine-specific histologic findings were correlated with the level of clinical rejection in a revised histological grading system.

Of the graft skin sections evaluated (n = 126), 15 were given Grade 0, 59 given Grade 1, 28 given Grade 2, 14 given Grade 3, and 10 given Grade 4 (Table 4). Along with inflammation, epidermal inflammatory cell infiltration and keratinocyte necrosis were recorded for each sample. With review of the samples, it was noted that not all specimens fit into the grading system outlined by the Banff criteria. Specifically, there were samples with significant inflammation but without epidermal infiltrates, and conversely, there were samples without significant inflammation but that did have epidermal infiltration. These characteristics were considered and stratified into subcategories within Grade 2 and Grade 3 of the proposed criteria. After full analysis of all of the samples, swine-specific trends and particular cellular characteristics were compiled to construct a new grading system for the skin component in swine VCA (Table 2).

Rejection Grade 0 consists of normal dermal and epidermal skin without evidence of inflammation. In swine

TABLE 2.
Swine VCA skin rejection classification (modified Banff criteria)

Grade	Dermal inflammation	Epidermal involvement
0	None to minimal	None
1	Mild	None
2A	Moderate	None
2B	Mild to moderate (may be paucicellular)	Infiltrating inflammatory cells (may be few) without keratinocyte necrosis
3A	Moderate or severe	Multifocal single cell epidermal necrosis, variable infiltrating inflammatory cells
3B	Mild to severe (may be paucicellular)	Multifocal epidermal necrosis (may be full thickness, not diffuse), infiltrating inflammatory cells
4	Mild to severe (may be paucicellular)	Diffuse full thickness necrosis (entire epidermis is necrotic and/or sloughed off)

VCA, vascularized composite allotransplantation.

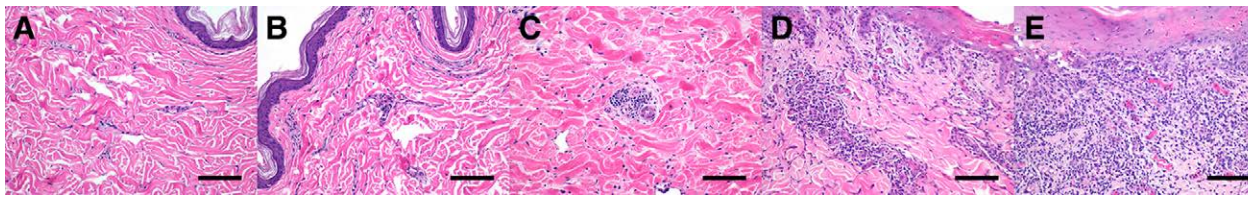

FIGURE 2. Examples of dermal inflammation scoring (a component of the proposed swine skin rejection scoring system). Inflammation scores are based on the following criteria: "none" (A), no perivascular cuffs of lymphocytes; "minimal" (B), <5 cuffs, ≤2 cells thick in any direction; "mild" (C), <5 cuffs, ≤2 cells thick in any direction; "moderate (D), 5–15 cuffs, any thickness; and "severe" (E), no distinct cuffs with diffuse infiltration, any thickness. The number of cuffs is determined by the average of the number of inflammatory cuffs counted over three ×20 fields in the dermis. All images are ×200 with 100 μm scale bars.

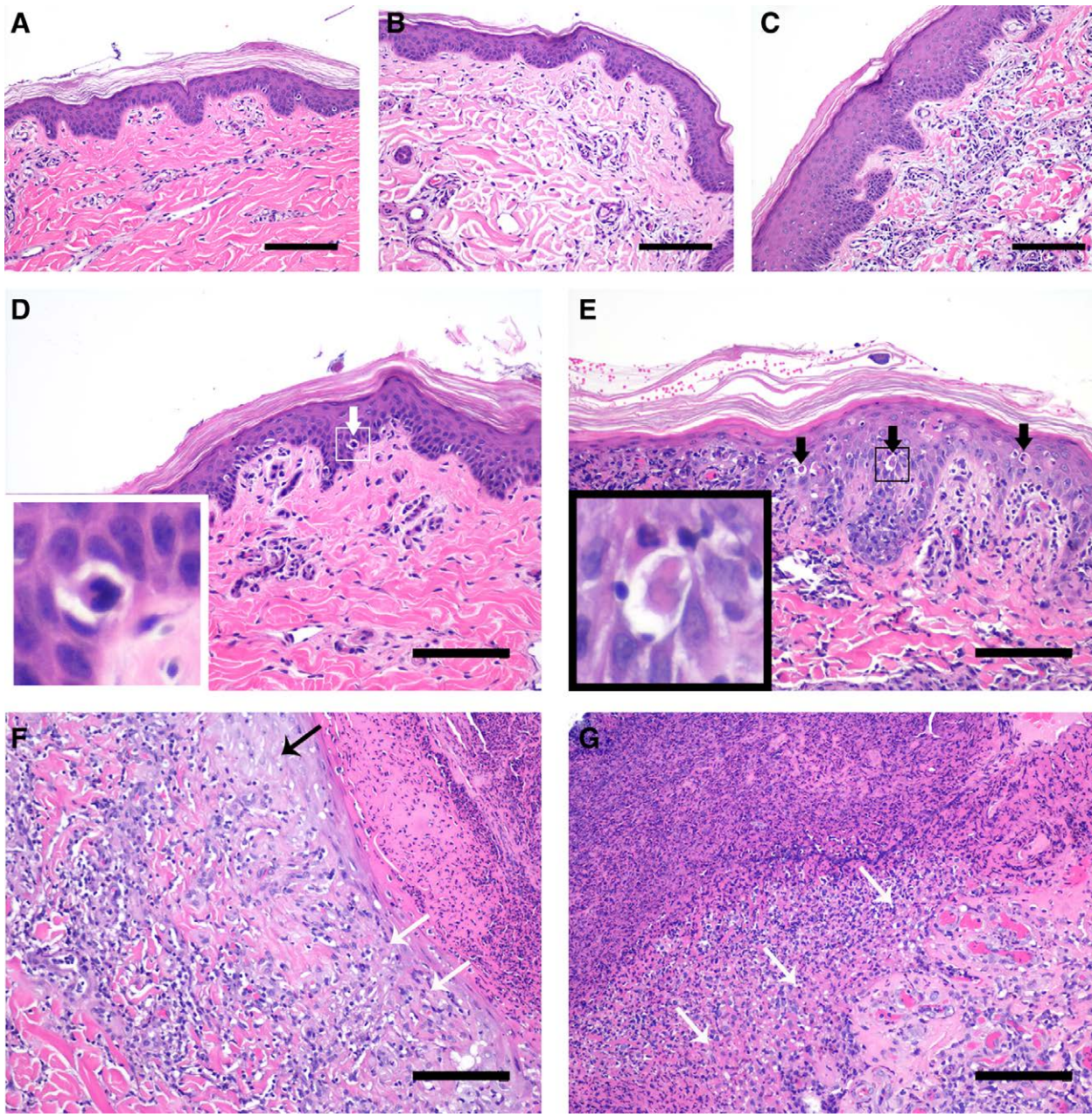

FIGURE 3. Examples of the proposed swine VCA skin rejection classification. Grade 0 rejection (A) and Grade 1 rejection (B) are characterized by none/minimal or mild inflammation (respectively) with no epidermal involvement. For the swine rejection classification, Grade 2 is split into 2A (C), characterized by dermal inflammation but no epidermal involvement, and 2B (D), characterized by variable inflammation with epidermal infiltrating inflammatory cells (white arrow, inset ×600). Grade 3 rejection is split into 3A (E), characterized by variable inflammation with single cell keratinocyte necrosis (black arrows, inset central arrow ×600), and 3B (F), characterized by multifocal or segmental full-thickness epidermal necrosis (white skinny arrows) with areas of intact epidermis (black skinny arrow). Grade 4 rejection (G), like in the original Banff classification, is characterized by diffuse full-thickness epidermal necrosis (white skinny arrows). All images are ×200 with 100 μm scale bars. VCA, vascularized composite allotransplantation.

TABLE 3.
Specimens reviewed

Specimen	Number
Ischemic controls	6
Native skin biopsies	88
Graft skin biopsies	120
Total samples reviewed	214

(as well as human) skin, there are always a small amount of perivascular lymphocytic infiltrates present in normal skin biopsies, which must be accounted for in giving rejection grades to allografts.[21] However, in Grade 0 rejection (Figure 3A), no epidermal changes are seen. Grade 1 (Figure 3B) also does not have epidermal changes; however, there is a mild perivascular lymphocytic infiltrate present, increased from the sparse lymphocytic infiltrate seen in normal porcine skin histology.

As previously mentioned, we have stratified Grade 2 rejection into two subcategories: Grade 2A (Figure 3C) and 2B (Figure 3D). This subdividing accounts for specimens that contain paucicellular perivascular inflammation but do have some epidermal infiltration without keratinocyte necrosis. Grade 2A is defined as moderate perivascular infiltrate based on cuff characteristics (Table 5) without epidermal involvement. The defining characteristic of Grade 2B rejection is the presence of epidermal inflammation; although there is often perivascular dermal lymphocytic inflammation, it can range from very few lymphocytes to moderate lymphocytic cuffing and accounts for up to but not necessarily moderate perivascular inflammatory cell presence with the aforementioned epidermal infiltration of inflammatory cells. Similarly, Grade 3 has been partitioned into 3A (Figure 3E) and 3B (Figure 3F). Rejection Grade 3A is characterized by moderate or severe inflammation with multifocal single cell epidermal necrosis. Grade 3B is characterized by variable dermal inflammation (up to severe) with multifocal, full-thickness, epidermal necrosis. Although both 3A and 3B feature epidermal necrosis, the key difference between the grades is that 3A has only single cell keratinocyte necrosis that does not affect the entire thickness of the epidermis (Figure 3E), while 3B has larger, multifocal areas of necrosis that involves the entire thickness of the epidermis, resulting in large areas of ulceration. However, in Grade 3B there are still areas of intact epidermis, while in Grade 4 the rejection is defined by diffuse, full-thickness, epidermal necrosis affecting the entire site.

For internal validation, all samples were also scored by a trained second independent, blinded party (M.G.), using the proposed porcine VCA skin rejection grades. A statistical analysis was performed to evaluate the discordance between the histological and clinical assessments of each sample. Given the subjectivity and lack of accepted standardization in grading of clinical rejection, association was evaluated in a dichotomous fashion using low-grade rejection, defined as Grades 0, 1, and 2, and high-grade rejection, defined as Grade 3 or Grade 4. A McNemar test was used to evaluate relationship between the low- and high-grade histologic and clinical rejection scores for each sample. Because the paired nominal data had few discordant pairs, a mid-p McNemar test was used.[22] The analysis resulted in a P value of 0.3, showing no evidence of discordance between the histologic and clinical grading systems.

Through the review of all specimens, graft capillary thrombosis was not appreciated. However, occasional occurrences of graft arteriopathy was noted, which were retrospectively found to be more frequent in those grafts that had been allowed multiple episodes of rejection (Figure S1, SDC, http://links.lww.com/TP/B713).

TABLE 4.
Characteristics of graft skin rejection assessments

	Histologic grade	Clinical grade
Grade 0	15	37
Grade 1	59	54
Grade 2	28	16
Grade 3	14	6
Grade 4	10	13
Total	126	126

TABLE 5.
Inflammation scoring rubric

Grade	Defining criteria: No. of perivascular cuffs of dermal lymphocytes ± macrophages and neutrophils/eosinophils (average over at least three ×20 fields)
None	No perivascular cuffs
Minimal	<5 cuffs, no more than 2 cells thick in any direction
Mild	<5, more than 2 cells thick in any direction
Moderate	5–15, any thickness
Severe	>15, any thickness

DISCUSSION

Experimental studies using swine models have been a staple in the preclinical study of VCA, due in part to the similarities between swine and human skin as well as the ease in operating on and assessing progress in this particular large animal model.[9] The Banff 2007 Working Classification for Vascularized Composite Tissue Allografts provided the first unified criteria for the grading of skin rejection in VCA in humans.[7] This classification greatly improved our ability as a field to compare and learn from other patients in this relatively rare procedure as well as to provide an objective measure to follow individual graft progression, assisting in both graft monitoring and titration of immunosuppressive treatment. However, while these criteria were also considered to be fairly applicable to the experimental swine models, as the skin is largely similar, there has not been an in-depth analysis of grading criteria as they pertain to histologic findings in swine skin. Given the importance of an accurate comparative model, we created these swine-specific grading criteria for skin rejection.

By retrospectively studying rejection in a large number of VCA transplants in MGH minipigs, we have proposed new, more refined rejection criteria specific to the MGH minipig, based on the original Banff criteria. Although we have highlighted many aspects of the striking similarity of pig skin anatomy and healing compared with that of human skin,[10-18] pigs are different than humans both in

their behavior and in some aspects of their inflammatory response. Pigs are more likely to traumatize skin posttransplantation, so small superficial pustules are not uncommon incidental findings. Anecdotally, pigs also have a more heavily eosinophilic component to their granulocytic inflammatory response compared to humans. However, the features of skin rejection, namely lymphocytic perivascular dermal inflammation and epidermal inflammation and necrosis, are strikingly similar.

The Banff 2007 Classification of skin rejection in VCA stratify the rejection grades by amount of inflammatory infiltrate present. Specifically, Grades 0 to 4 histologic rejection are defined in part by no or rare inflammation, mild inflammation, moderate inflammation, severe inflammation, and necrosis, respectively.[7] In our proposed new grading criteria, we have subdivided rejection Grades 2 and 3 into 2A/2B and 3A/3B. Within Grade 2 rejection, we have distinguished between rejection characterized by moderate dermal inflammation without epidermal involvement and rejection characterized by variable dermal inflammation but inflammatory cell infiltration of the epidermis. This delineation is important, as in our experience, epidermal cellular involvement tends to correlate better with worse clinical rejection when compared to strictly dermal perivascular inflammation despite paucicellular inflammation that may not correlate with the moderately cellular inflammation assigned to Grade 2. For Grade 3, we have distinguished between epidermal necrosis that is single cell (3A) versus numerous (3B), accounting for the possibility of multifocal epidermal necrosis with different levels of inflammatory infiltration. Where the Banff 2007 Criteria defines Grade 3 histologic rejection by dense inflammation with epidermal involvement, we have noted in swine rejection that fairly severe epidermal necrosis may be associated with relatively few inflammatory cells in the dermis, and yet still quickly progress to Grade 4 rejection. This necessitated a subdivision of Grade 3 rejection that included more severe epidermal necrosis with or without large perivascular lymphocytic cuffs in the dermis. With this new definition of Grade 2 and 3 rejection in VCA skin in a swine model, we can accurately place histopathological grades by biopsy including the details that might otherwise have assigned other grades to these specimens.

Using our revised, swine-specific rejection criteria, we have drawn several conclusions from acutely rejecting animals both in general pattern and in specific details. Although granulocytes (neutrophils and eosinophils) as well as macrophages were present in rejecting skin samples, the vast majority of infiltrating inflammatory cells were lymphocytes. Of these, most were T cells, with fewer B cells (Figure S2, SDC, http://links.lww.com/TP/B713), consistent with previous findings.[5,23,24] While overall inflammation is a major component of our modified grading criteria, the most clinically relevant factor seems to be the extent of inflammatory infiltration into the epidermis. Similar to human VCA rejection, our group found that swine grafts could be rescued up to but not including Grade 4 histopathologic rejection, which is characterized by diffuse epidermal necrosis.[7] Notably, even those that had histologic Grade 3B rejection—with multifocal epidermal necrosis—were able to be rescued using standard immunosuppressive treatment (steroid bolus treatment and calcineurin inhibitor) due to the ability of the graft to reepithelialize. We also found that dermal inflammation could be quite significant, but if the epidermis was not involved, the clinical appearance was much less severe with a relatively low clinical rejection score (Grade 2 or lower) (Figures 1 and 3). We also did not include inflammation in the subcutis in the rejection scoring system, as subcuticular inflammation does not reflect the appearance or behavior of the graft; clinically important inflammation is restricted to the dermis and epidermis. Neutrophilic inflammation was significantly correlated with Grade 4 rejection (Figures 2 and 3). However, neutrophilic dermatitis is not considered specific to the pathogenesis of rejection; rather, neutrophils are a generic response to tissue damage (in this case, epidermal necrosis).[25,26]

Through this extensive review of pathologic specimens, it became increasingly evident that the accurate assessment of rejection and grading relies not only on a good grading system but also on the technical aspects of obtaining, preserving, and staining the biopsy as well. When evaluating graft rejection, it is important to interpret the histologic appearance in the context of the gross appearance. Ideally, multiple biopsies should be obtained from multiple sites. Significant differences in histologic appearance can occur within the same graft even millimeters apart. The clinical rejection of an experimental graft should not necessarily be predicted based on one punch biopsy taken from a focal area of epidermal necrosis, as reepithelialization of the necrotic area may occur if the rest of the graft survives and the necrotic area is small. Specimen preparation is also of importance, as the maintenance of tissue architecture is relevant to enable slide staining and get high quality, consistent specimens to evaluate pathologically. Though we did have some excluded samples for which the biopsy and/or fixing or embedding provided slides with insufficient tissue to adequately assess, the rest of our samples were uniform enough that they could be adequately compared. However, in our experience, 24 hours of formalin fixation followed by placing the sample in ethanol before paraffin embedding provided the optimal preparation.

As mentioned previously, it can be difficult to ensure that these grafts remain without scratching or traumatic injury, as this can cause inflammation unrelated to rejection that can confound histologic appearance. The grafts are insensate, so preventing the animal from injuring the graft requires diligence and attention. For this purpose, our included animals were all maintained in single-animal runs after transplantation to avoid graft damage from another pig. The cohort was also housed in specialized runs with protective polyethylene paneling that provides smooth walls to the enclosure. The animals are seen at least once a day to assess graft condition. This prevents the majority of animal scratching of the graft in our studies and largely mitigates the concern for inflammation unrelated to rejection. Furthermore, we do not currently have complete knowledge on the effect of the experimental treatment regimens on the skin and histological outcomes. Most of the animals received tacrolimus therapy either for a set time period or in pulsed dosing, though a few had costimulation blockade or cellular therapy. None of the regimens correlated with any particular rejection grade, but as our study evaluated skin samples only in the context of whether or not they were rejecting and independent of the individual treatment regimens, we cannot exclude confounding of the different experimental treatments on the skin rejection grade.

The importance of this proposed grading system lies in its implications for future studies. As the histologic grading system shows correlation to the clinical grading system, it can be used in the setting of an acute rejection episode to delineate the severity of the episode, often not homogenous throughout the graft. However, with accurate grading of acute rejection episodes, we can also evaluate the relationship between acute rejection clinical appearance, the grade of acute rejection, and the development of chronic rejection changes and other long-term outcomes. Graft arteriopathy was noted in several specimens, particularly in those that experienced multiple episodes of acute rejection (**Figure S1, SDC**, http://links.lww.com/TP/B713), possibly representing chronic changes. It has been shown that increased number of acute rejection episodes is associated with increased risk of chronic rejection[27,28]; while this has not been studied in depth in translational models, an accepted and reproducible grading system for the acute episodes will prove important in a thorough investigation into this topic.

In the current era of rapid medical and surgical advancements, adequate preclinical models are crucial to continued medical research and patient safety. Because of the limited patient population in the relatively young field of VCA, preclinical models are even more critical to our understanding of the relevant immunomodulatory processes and our discovery of less toxic and more effective treatment regimens. These new criteria defined here for histologic grading of skin rejection in swine—with the grading criteria paralleling those of the Banff Classification—provide a uniformity in histopathological assessment and contribute to the ability to analyze findings in swine preclinical models in the evaluation of VCA rejection.

ACKNOWLEDGMENTS

We acknowledge the contributions and assistance in the large animal operations of the entire Johns Hopkins VCA Laboratory team as well as our veterinarian colleagues—specifically Dr. Jessica Izzi and Dr. Caroline Garrett—and the veterinary technicians Melanie Adams, Kristy Koenig, and Gabriella Dancourt. We acknowledge the support of the Army, Navy NIH, Air Force, VA and Health Affairs regarding the AFIRM II effort under award CTA05: W81XWH-13-2. The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702–5014 is the awarding and administering acquisition office. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense. In addition, we acknowledge support of the FY12 Restorative Transplantation Research (RTR) program under award W81XWH-13-2-0060 MR120034P10.

REFERENCES

1. Mathes DW, Randolph MA, Solari MG, et al. Split tolerance to a composite tissue allograft in a swine model. *Transplantation.* 2003;75(1):25–31.
2. Sinha I, Pomahac B. Split rejection in vascularized composite allotransplantation. *Eplasty.* 2013;13:e53.
3. Starzl R, Brandacher G, Lee WP, et al. Review of the early diagnoses and assessment of rejection in vascularized composite allotransplantation. *Clin Dev Immunol.* 2013;2013:402980.
4. Wolfram D, Starzl R, Hackl H, et al. Insights from computational modeling in inflammation and acute rejection in limb transplantation. *PLOS One.* 2014;9(6):e99926.
5. Sarhane KA, Khalifian S, Ibrahim Z, et al. Diagnosing skin rejection in vascularized composite allotransplantation: advances and challenges. *Clin Transplant.* 2014;28(3):277–285.
6. Hautz T, Zelger BG, Weissenbacher A, et al. Standardizing skin biopsy sampling to assess rejection in vascularized composite allotransplantation. *Clin Transplant.* 2013;27(2):E81–E90.
7. Cendales LC, Kanitakis J, Schneeberger S, et al. The banff 2007 working classification of skin-containing composite tissue allograft pathology. *Am J Transplant.* 2008;8(7):1396–1400.
8. Etra JW, Raimondi G, Brandacher G. Mechanisms of rejection in vascular composite allotransplantation. *Curr Opin Organ Transplant.* 2018;23(1):28–33.
9. Brandacher G, Grahammer J, Sucher R, et al. Animal models for basic and translational research in reconstructive transplantation. *Birth Defects Res C Embryo Today.* 2012;96(1):39–50.
10. Pfützner W, Joari MR, Foster RA, et al. A large preclinical animal model to assess ex vivo skin gene therapy applications. *Arch Dermatol Res.* 2006;298(1):16–22.
11. Laurent PE, Bonnet S, Alchas P, et al. Evaluation of the clinical performance of a new intradermal vaccine administration technique and associated delivery system. *Vaccine.* 2007;25(52):8833–8842.
12. Cuttle L, Kempf M, Phillips GE, et al. A porcine deep dermal partial thickness burn model with hypertrophic scarring. *Burns.* 2006;32(7):806–820.
13. Dincer Z, Jones S, Haworth R. Preclinical safety assessment of a DNA vaccine using particle-mediated epidermal delivery in domestic pig, minipig and mouse. *Exp Toxicol Pathol.* 2006;57(5–6):351–357.
14. Klíma J, Lacina L, Dvoránková B, et al. Differential regulation of galectin expression/reactivity during wound healing in porcine skin and in cultures of epidermal cells with functional impact on migration. *Physiol Res.* 2009;58(6):873–884.
15. Jiang H, Zhang HM, Frank MM. Subcutaneous infusion of human C1 inhibitor in swine. *Clin Immunol.* 2010;136(3):323–328.
16. Wempe MF, Lightner JW, Zoeller EL, et al. Investigating idebenone and idebenone linoleate metabolism: in vitro pig ear and mouse melanocyte studies. *J Cosmet Dermatol.* 2009;8(1):63–73.
17. Debeer S, Le Luduec JB, Kaiserlian D, et al. Comparative histology and immunohistochemistry of porcine versus human skin. *Eur J Dermatol.* 2013;23(4):456–466.
18. Summerfield A, Meurens F, Ricklin ME. The immunology of the porcine skin and its value as a model for human skin. *Mol Immunol.* 2015;66(1):14–21.
19. Ibrahim Z, Cooney DS, Shores JT, et al. A Modified Heterotopic Swine Hind Limb Transplant Model for Translational Vascularized Composite Allotransplantation (VCA) Research. *J Vis Exp*. 2013;(80):e50475.
20. Schneeberger S, Khalifian S, Brandacher G. Immunosuppression and monitoring of rejection in hand transplantation. *Tech Hand Up Extrem Surg.* 2013;17(4):208–214.
21. Kanitakis J. Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol*. 2002;12(4):390–309; quiz 400–401.
22. Fagerland MW, Lydersen S, Laake P. The mcnemar test for binary matched-pairs data: mid-p and asymptotic are better than exact conditional. *BMC Med Res Methodol.* 2013;13:91.
23. Wolfram D, Morandi EM, Eberhart N, et al. Differentiation between acute skin rejection in allotransplantation and T-cell mediated skin inflammation based on gene expression analysis. *Biomed Res Int.* 2015;2015:259160.
24. Kirk AD, Madsen JC, Larsen CP, et al. Ch 87: Histopathological syndromes of vascularized composite allograft rejection and recurrent disease. In: Kirk AD, Knechtle SJ, Larsen CP, Madsen JC, Pearson TC, Webber SA, eds. *Textbook of Organ Transplantation*. 1st ed. Chinchester, UK: Wiley-Blackwell; 2014:1023–1029.
25. Su Y, Richmond A. Chemokine regulation of neutrophil infiltration of skin wounds. *Adv Wound Care (New Rochelle).* 2015;4(11):631–640.
26. Kim MH, Liu W, Borjesson DL, et al. Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. *J Invest Dermatol.* 2008;128(7):1812–1820.
27. Unadkat JV, Schneeberger S, Horibe EH, et al. Composite tissue vasculopathy and degeneration following multiple episodes of acute rejection in reconstructive transplantation. *Am J Transplant.* 2010;10(2):251–261.
28. Kanitakis J, Karayannopoulou G, Lanzetta M, et al. Graft vasculopathy in the skin of a human hand allograft: implications for diagnosis of rejection of vascularized composite allografts. *Transpl Int.* 2014;27(11):e118–e123.