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SURVIVAL OF ALLOGENEIC SELF-ASSEMBLED CULTURED SKIN

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Abstract

Background—Deficiency of autologous skin for reconstruction of severe wounds is a major problem in plastic surgery. Autologous substitutes can provide additional coverage, but due to the duration of production, treatment is significantly delayed. The allogeneic approach offers a potential of having an off-the-shelf solution for the immediate application.

Methods—In this study, we assess the engraftment and immunogenicity of allogeneic bilayered bioengineered skin prepared by a self-assembly method. Bioengineered skin has the potential immunological advantage of lacking passenger leukocytes including antigen presenting cells. The skin constructs were transplanted across MHC barriers in a porcine animal model. Animals received a second grafting of the same skin construct 7 weeks following the first set of grafts together with MHC-matched constructs to assess for clinical sensitization.

Results—All allo-constructs successfully engrafted with histologic evidence of neovascularization by day 4. Complete cellular rejection and tissue loss occurred by day 8 for most grafts. Following the second application, accelerated rejection (<4 days) took place with the development of swine MHC-specific cytotoxic alloantibody.

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Conclusions—These data demonstrate preclinically that self-assembled allogeneic constructs engraft and reject similar to allogeneic skin despite the absence of professional donor antigen-presenting cells.

INTRODUCTION

Skin is the largest organ of the body, the major interface with the outside world, and it plays the central role in host defense(1). It represents the best reconstructive mean for many complicated injuries, including burns(2). Such wounds are typically treated with autologous split-thickness skin grafts (STSG), harvested from a separate anatomical site on the body. When sufficient amounts of skin are not available because of extensive resections, trauma or large defects, alternative solutions are required(3). Most alternative solutions, including cadaveric skin, xenografts, and bioengineered constructs, reject and require additional skin grafting, or do not engraft and function mostly as biological dressings providing only temporary coverage. Existing cellularized engineered skin substitutes offer sub-optimal rates of engraftment and require multiple applications at a significant cost(4).

Self-assembled bioengineered skin, termed skin construct (SC), derived from autologous dermal fibroblasts and keratinocytes isolated from a skin punch biopsy and assembled into dermal and epidermal layers following stimulation to produce an extracellular matrix (ECM), was shown in a swine model to engraft and persist indefinitely(5). Lack of foreign proteins and the presence of a self-derived ECM may have contributed to timely engraftment and prevention of contraction. However, the length of production, the expense and the necessity of skin punch biopsy would limit large-scale usage of this technology. Development of a “universal” skin substitute that would be accepted by all patients and could be produced on an industrial scale is desirable. In our opinion, the best suitable way to achieve this and to avoid the graft donor site morbidity is to use allogeneic or xenogeneic approaches. Attempts of allo-skin transplantation have been made throughout history. However, the aggressive rejection and sensitization, which allow single-use only of the allo-skin limit its clinical application(6). Such sensitization would also make it difficult to find a suitable donor in the case in which a reconstruction with vascularized composite allograft may be indicated. Skin is a vital, highly active immunological organ that continuously provides communication with the environment(1). Many studies have been performed to understand skin immunity since Medawar’s successful skin transplantation, and much progress has been achieved; however, the mechanisms of rejection remain unclear. Understanding the process of skin transplant rejection could lead to novel approaches for induction of clinical skin tolerance(7). Expression of MHC class II on professional passenger antigen presenting cells (APC) within the donor skin is thought to play a critical role in skin rejection(8,9). Self-assembled SC previously introduced by our team lacks not only the foreign ECM by design but also lacks professional APCs. This fact may facilitate persistence or delay rejection in an allogeneic situation. We tested this hypothesis in translational, preclinical large animal model across MHC barriers. The SC were generated from dermal fibroblasts that were stimulated to produce an ECM and were then seeded with cultured keratinocytes to generate an epidermis, as described in Material and Methods. The construct derived in this fashion was subsequently transplanted across a full-2 haplotype

MHC barrier in swine. This type of mismatch leads to vigorous rejection of allografts in the absence of immunosuppression(10–13).

MATERIAL AND METHODS

Animals

MGH MHC-defined miniature swine(14,15) were used in this study. Animals were housed at the Transplantation Biology Research Center during the experiments. All experimental procedures were carried out in accordance with the Guide for Care and Use of Laboratory Animals (Eighth edition, National Academy of Sciences Press, 2011), and were approved by the Institutional Animal Care and Use Committees of the Massachusetts General Hospital (MGH). The swine used in this study were inbred for the MHC and outbred for minor histocompatibility antigens (MiHAs). Six MGH Swine Leukocyte Antigen CC (SLA^{cc}) animals age of 5–7 months were used as recipients. Five animals received SLA^{dd} SC (DD-SC), and 1 animal received a SLA^{dd} STSG (DD-STSG) to constitute a full 2-haplotype mismatch combination (Figure 1) which induces vigorous rejection of the allograft(10,12,13). Animals (n=6) underwent 2 consecutive surgeries 7 weeks apart. Five animals received 2 DD-SC and 1 animal received 2 DD-STSG during the first surgery. During the second surgery, all animals underwent repeated application of the same MHC-mismatched DD-SC/STSG placed during the first surgery together with an MHC-matched, MiHA-mismatched CC-SC/STSG. The grafting scheme is shown in Table 1. The animal that received MHC-mismatched DD-STSG (21259) was used as a positive control and exhibited rejection at a comparable timepoint (6 days) as historical MHC-mismatched STSG (7 +/- 0.36 days) as described in detail previously(10). At each surgery time-point, all the animals received 2 autologous STSGs to control the grafting technique and provide specimens for comparison.

SC generation

Six mm skin punch biopsies from neonatal (0–3 days) MGH MHC-defined swine of SLA^{dd} and SLA^{cc} haplotypes were taken in sterile conditions under local anesthesia using punch biopsy knife. Punch biopsies were incubated with trypsin and collagenase, and cells were collected by centrifugation. Cells were then serially passaged in media favoring the proliferation of either fibroblasts or keratinocytes described previously(16), except that the keratinocyte culture medium, MSBM, was modified by the addition of triiodothyronine, bovine pituitary extract and cholera toxin to prevent differentiation during expansion. Cultures were cryopreserved at 2 stages to create master cell banks and working cell banks. Porcine SC were generated from the fibroblast and keratinocyte working cell banks at the Preclinical Research and Development Laboratory, Organogenesis Inc. (Canton, MA). A dermal matrix was prepared from dermal fibroblasts stimulated to generate an ECM, and then seeded with keratinocytes to form an epidermal layer as described by Pouyani et al.(17–20), except that fibroblasts were cultured in DMEM containing 20% fetal bovine serum and 50 µg/mL L-ascorbic acid-2-phosphate. The SC were constructed in a 75 mm tissue culture insert (Corning Incorporated Life Sciences, Tewksbury, MA). Batches of SC underwent quality control testing at the Organogenesis facility before grafting (Figure S3), to ensure sterility, morphology, and viability.

STSG and SC grafting

A partial-thickness wound model was used in this study, due to its consistent depth and a documented rejection of the allogeneic skin grafts(21,22). Animals were anesthetized with Telazol, Glycopyrrolate, intubated and maintained with isoflurane (1–3%)/oxygen. Animals were placed in the prone position. The back was shaved, prepped and draped in a typical aseptic fashion. Four wounds (2×2 inch, 2 per side) were created with a dermatome on the back of the swine until punctuate bleeding was observed, by the same operators (MC, MR). A saline solution of 1:10000 (Epinephrine) and compression was applied for the hemostasis. Two wounds in each animal were treated with autologous STSG to characterize wound bed vascularity/quality of the wound bed and the technique of grafting. Another 2 wounds were treated with DD-SC. STSG were fenestrated with a scalpel in an interdigitated fashion, and with the SC applied to the designated wounds, anchored with Prolene 4-0. SC were fenestrated after they were anchored (Fig. S1). Photographs (Sony Cyber-shot DSC-TX10) of the wounds were taken before and after grafting. Petrolatum gauze (Adaptic-Systagenix, San Antonio, TX, USA), 2 gauzes in a wet to moist way, KCI V.A.C. GranuFoam (KCI, San Antonio, TX, USA) precut in squares 5×5 cm were sutured to the wound with 3-0 silk sutures and covered with Tegaderm (3M Healthcare-St. Paul, MN, USA), VetWrap and stockinette. After 7 weeks, the animals underwent a second surgery to create 6 (3/side) partial-thickness excisional wounds on their dorsal surface at sites caudal to the initial wound sites (none of the wounds was created on the previous areas of graft placement). Animals underwent a second grafting of DD-SC along with CC-SC to assess for clinical sensitization. Two wounds in each animal were treated with autologous STSG to characterize wound bed vascularity/quality of the wound bed and the technique of grafting except animal #20863, where 1 of the wounds was grafted with a minor mismatched SLA^{cc} STSG (from animal #20839, Table 1). Engraftment and course of rejection were compared to that of the minor mismatched SLA^{cc} construct.

Graft biopsy

A total of 524 punch biopsies were taken and analyzed for this study. Two biopsies were taken from each graft on days 4, 6, 8, 10 and 12 after each surgery. Biopsies were fixed in 10% neutral buffered formalin for subsequent paraffin embedding. Paraffin-embedded sections were stained with H&E for evaluation of tissue morphology, immunohistochemistry, or CD31 (Clone SP164; Spring Bioscience, Pleasanton, CA, USA). Sections were observed on a Zeiss Axio Imager 2 (Carl Zeiss Microscopy GmbH, Goettingen, Germany) and images were recorded at 10× magnification using AxioVision software (Carl Zeiss Microscopy GmbH, Munich, Germany) and ImageScope v10 (Leica Biosystems, Buffalo Grove, IL, USA). All paraffin embedding and tissue sectioning of laboratory research material was performed by the MGH Department of Pathology. A dedicated pathologist (EAF) analyzed the sections blinded, graded them according to preestablished criteria and created a database in FileMaker (FileMaker Inc., Santa Clara, CA, USA). Engraftment or rejection was evaluated by clinical appearance and by histology.

Assessment of Immunogenicity

Serum was collected weekly to assess titers of circulating anti-donor MHC or anti-self MHC antibodies. The antibody response to DD-SC and DD-STSG was evaluated by flow cytometry using serum obtained before and at different time points after grafting. The presence of anti-donor alloantibody was assessed by flow cytometry-based binding and complement-dependent cytotoxicity assays(11,23,24). Peripheral Blood Mononuclear Cells (PBMC) were prepared by Ficoll gradient centrifugation(23).

Antibody binding assay

Donor-matched (SLA^{dd}) and recipient-matched (SLA^{cc}) PBMC were incubated with the serum obtained from recipient animals at different time points, stained as described by Cho et al(11), and then assessed by flow cytometry.

Complement-Mediated Antibody Dependent Cellular Cytotoxicity Assay

Allogeneic cytotoxic antibodies to donor PBMCs were detected by complement-mediated cytotoxic assays, as described previously(24). Briefly, target cells (recipient and donor type) were resuspended at a concentration of 5×10^6 cells/ml in Medium 199 (Cellgro, Herndon, VA, USA) supplemented with 2% fetal calf serum, and were incubated for 15 min at 37 °C in 96-well U-bottom plates with 25 μ l of serum, serially diluted from 1:2 to 1:1024, or with controls (Costar, Cambridge, MA, USA). A second incubation was then carried out with 25 μ l of appropriately diluted rabbit complement. The percentage of dead cells were identified by staining with 10 μ l of 7-AAD for 30 min. For the anti-MHC class II DD assay (Figure 6), the serum was incubated with SLA^{GG} PBMC (MHC class I of C and class II of D) (Figure 1A), thus only antibodies anti-class II would bind the target cells and subsequently allow lysis by complement.

Flow cytometry

The following swine specific antibodies were used: in-house anti-class I^c (16.7.E4.2) and anti-class I^d (2.12.3a), CD172a (74-22-15), MHC class I (2.27.3), MHC class II DQ (TH16), MHC class II DR (40D). Data were acquired using Becton–Dickinson FACScan (San Jose, CA, USA) and analyzed with WinList analysis software (Verity Software House, Topsham, ME, USA) and FlowJo version 8.8.6.

Tissue digestion

STSG and SC obtained from SLA^{dd} animal 20687 (the construct that was used for grafting on all SLA^{cc} recipient animals) were enzymatically dissociated and with fresh isolated PBMC (20687) stained and analyzed by flow cytometry. STSG and SC were macerated with a scalpel and underwent tissue dissociation with collagenase as described by Clark et al(25). Briefly, the sample was washed twice in HBSS and minced with scissors and incubated for 2 hours at 37°C with continuous shaking at 200 RPM in collagenase type 1 and DNase. Finally, the sample was filtered through 70 μ m nylon mesh, washed with HBSS and the cell counts were adjusted for the flow cytometry to assess the expression of MHC.

Image acquisition and processing

Photos were acquired with Sony Cyber-shot DSC-TX10 - digital camera, figures were created using Microsoft Office 2010, Adobe Photoshop 10.0 and Adobe InDesign version 5.0 (Adobe Systems Incorporated, San Jose, CA, USA)

RESULTS

Tissue dissociated SC did not express MHC class II and showed a lower expression of MHC class I compared to PBMC and dissociated STSG (Figure 2). A total of 60 grafts (SC, autologous STSG as depicted in Table 1) were placed in this study, from which none failed. No significant infection, contamination, hematoma or seroma was noticed. A total of 10 MHC-mismatched DD-SC were grafted on 5 SLA^{cc} animals during the first surgery (Figure 1B, Table 1). A small degree of shearing/contraction was observed in wounds treated with the SC (Figure 3B-POD4). All autologous control STSG took without complications (n=10). All SC successfully engrafted with histological evidence of neovascularization of the fibroblastic component by POD 4 (Figure 3B, C). By POD 6, perivascular inflammatory infiltrates were found in the fibroblastic layer; some with extension to the epidermal layer (grades 1–2 by Banff classification of skin containing composite tissue allograft pathology(26) (Figure 3D, S2). Complete cellular rejection and tissue loss occurred by POD 8 for most grafts. Following the second application, both DD-SC and CC-SC (full MHC-mismatched and MiHAs-mismatched, respectively) underwent accelerated rejection (<4 days) (Figure 4). Pathological findings showed angiogenesis during first grafting and lack of it after regrafting (Figure 3C, 4C, D). Donor MHC-specific alloantibody could be detected within 10–18 days post first grafting, with increased titer post regrafting (Figure 5A). Formed anti-MHC antibodies were shown to be cytotoxic (Figure 5B). Donor-specific MHC class II DD cytotoxic antibodies could not be detected following rejection in any of the 5 recipients of MHC-mismatched SC (Figure 6) confirming the lack of donor MHC class II positive cells within the construct. In contrast, the control animal (21259), which rejected DD-STSG by day 6, developed donor specific MHC class II DD cytotoxic antibodies, as expected (Figure 6). MiHA STSG placed on animal 20863 along with MiHA SC, and MHC-mismatched SC placed for the second time experienced poor engraftment and rejected clinically and histologically by day 6 (Figure S4).

DISCUSSION

The importance of MHC class II positive professional APC for the development of the immune response after transplantation is well known(8,9,27). Based on experience with Dermagraft(28), and Ferreira's hypothesis(2), it was hopeful that an artificial skin lacking professional APC may persist without sensitization to the donor MHC. This study confirms that eliminating all but fibroblasts and keratinocytes from skin is not sufficient to allow acceptance of the skin in swine, similar to results shown previously by Auchincloss, where class II-deficient skin was rejected by normal mice(29). Our study reinforces the findings of Burt et al(21) that long-term survival of allogeneic cultured keratinocyte sheets is not possible even in a combination with so-called "tolerizing" dermal fibroblasts(28,30). However, as a temporary solution, cultured keratinocyte allografts have been used for

chronic wounds as shown by Phillips and Gilchrist(31). In this case, the sheet of allogeneic keratinocytes served as an active biological dressing by secreting growth factors and cytokines that promote wound healing(31). Also it is important to note that skin rejection is a clinical diagnosis made when over 90% of the graft is lost as measured by clinical judgement of the operator. Assessment in our study was performed only on days 4,6,8,10,12 skipping the days between, due to the set dressings change points. The 1 animal reported to reject by day 10 actually rejected sometime between 8–10 days.

The fact that accelerated rejection occurred following a second placement of the initial construct confirms sensitization clinically and the employment of the second-set rejection. It is unclear why MHC-matched (MiHAs mismatched) SC, placed for the first time during the 2nd surgery, rejected in an accelerated fashion. One possibility is that both allogeneic SC (SLA^{dd} and SLA^{cc}) shared MiHAs, presensitization to which caused the accelerated rejection. It was shown previously that MiHAs can be responsible for aggressive rejection of skin grafts, and that MiHAs are as important as MHC in skin rejection(32,33). Alternatively, there may be other as yet unknown cell populations present in normal skin but missing from SC which dampens immune responses to MiHAs. Also important to note is that the fact of biopsying each wound could potentially induce inflammation sufficient to initiate the rejection process.

It is known that keratinocytes can present antigen, but only in inflamed conditions(34,35), which may be the case in the partial-thickness wound model used in our study. However, keratinocytes have also been reported to be tolerogenic when not inflamed(36). It was shown as well that a non-professional APC, such as endothelial cells, can express MHC class II molecules that are sufficient to mediate rejection(37,38). In contrast to animals that reject allogeneic STSG, no anti-MHC class II antibodies were detected in animals that rejected allogeneic SC lacking APCs. This implies sensitization through the indirect pathway in which the donor antigens were processed and presented by the recipient professional APC following graft vascularization (Figure 7).

It is important to emphasize that even in the allogeneic situation the SC can engraft and vascularize similarly to the autologous SC(5). Despite the rejection in our model, the allogeneic SC could be potentially used for the study of skin immunity. Different cell populations could be introduced to research the tolerance of skin. The swine animal model presents significant advantages over the murine models due to the closer resemblance of the skin and the immune system to humans(39,40). Moreover, the rejection of the allogeneic SC similarly to the cadaveric allograft, suggests potential usage of such constructs for temporary coverage.

The fact that anti-donor alloantibodies were detected following the rejection of the initial construct demonstrates that T cell priming did occur.

It should be noted that other bioengineered constructs use allogeneic fibroblasts and keratinocytes and that the bilayered living cellular construct (BLCC, Apligraf®) resembles the SC structurally. However, although the BLCC is applied under allogeneic conditions, it does not engraft or sensitize the recipient like the allogeneic SC(41,42). Vascularization of

the SC likely drives the allosensitization of the recipient, whereas the BLCC does not vascularize, nor sensitize the recipient.

Our findings are important because we introduce a new tool for skin immunology and tolerance research, this helps in understanding the skin rejection process and adds to the pursuit of ideal skin substitute. Our data demonstrates that the lack of professional APC in the skin does not, by itself, prevent rejection or prolong survival and that the autologous ECM promotes engraftment even in allogeneic situation.

In summary, these data demonstrate that allogeneic SC engraft, undergo vasculogenesis and eventually reject similar to allogeneic skin grafts. Despite the lack of donor hematopoietic-derived cells, including MHC class II⁺ APCs, allogeneic SC is rejected in a similar time course to allogeneic skin and sensitize across MHC and MiHA barriers. Since the absence of professional donor-APC does not appear to prolong graft survival, sensitization to minor histocompatibility antigens likely occurred through indirect antigen presentation following vascularization and host cell infiltration into the construct.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ECM	extracellular matrix
SC	Skin Construct (dermal and epidermal artificial skin generated by self-assembly, with dermal component derived by stimulating fibroblast to secrete ECM) transplanted as autologous or across MHC and/or MiHAs barriers
SLA	Swine Leukocyte Antigen (porcine MHC)
STSG	Split-Thickness Skin Graft (can be autologous, allogeneic or xenogeneic)
MiHA	Minor Histocompatibility Antigens
APC	Antigen Presenting Cells

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Figure 2.

Skin (middle panel) and Skin Construct - SC (right panel) from animal 20687 (animal that donated cells for the DD-SC used in the entire study) were digested with collagenase/DNase, stained, phenotyped by flow cytometry and compared with peripheral blood mononuclear cells (PBMC) from the same animal (shown in the left panel). No expression of MHC class II and low expression of MHC class I were found in the digested SC. Lower panels: closed histogram-isotype controls, open histogram-MHC class I antibody.

Figure 3.

Engraftment of the allogeneic SC and subsequent rejection vs. rejection of the allogeneic STSG. **A.)** Clinical appearance of the successive time points of DD-STSG (not construct) rejection (animal #21259), **B.)** Corresponding time points (to A.) of the DD-SC rejection, on POD8 the construct sloughed off leaving naked wound bed. The first image in the row (POD4) emphasizes a small degree of shearing (left edge). **C.)** Histological section of the formalin-fixed DD-SC-treated wound on POD4 showing angiogenesis toward the dermal-epidermal interface. **D.)** Polymorphonuclear perivascular infiltrate of the neo-formed vessels on POD6 of the DD-SC treated wound. **E.)** POD6 of the DD-STSG treated wound shows non-viable graft with massive cellular infiltrate (C, D, E 20x, HE staining).

Figure 4.

Accelerated rejection of SCs during second surgery. Serial time points are shown. Starting with POD4 both constructs: MHC-matched, MiHA mismatched SC placed for the 1st time (A), and MHC-mismatched SC placed for the 2nd time (B) show visual and histological signs of non-engraftment/early rejection (C, D). Although the SC is present on the wound (in both cases), it has a slightly wet appearance, the fenestrations do not appear to close and starting with day 8 the wound shows signs of healing (hair growth, sloughing and crusting), this suggest non-engraftment, early rejection, and secondary intention healing, completely different aspect than it can be observed in Figure 3. C) H&E histology emphasizing lack of engraftment and cellular infiltration of the wound bed of the CC-SC on POD4. D) H&E histology emphasizing lack of engraftment and cellular infiltration of the wound bed of the DD-SC on POD 4 (completely different from Figure 3)

Figure 5.

Anti-donor alloantibodies could be detected after the rejection of the MHC-mismatched SC by flow cytometry (**A**) and by antibody-dependent, complement-mediated cytotoxicity assays (**B**). Anti-donor antibodies were specific and manifested anti-donor MHC cytotoxicity but not anti-recipient MHC (all animals shown in **A** and representative animal #20668 shown in **B**).

Figure 6.

Donor-specific (anti-SLA^{dd}) class II antibodies are induced following the rejection of allogeneic STSG (in animal 21259), but could not be detected in any of the 5 recipients of allogeneic SC following rejection. Serum at different time points was incubated with GG pbmc (MHC class I of SLA^{cc} and MHC class II of SLA^{dd}), and complement was added thereafter as described in the materials and methods. If anti-donor MHC class II antibodies were present in the serum, this would lead to cell lysis.

Figure 7.

Diagram depicting the potential mechanism of sensitization and skin construct (SC) rejection. With the ingrowth of blood vessels (days 3–4) antigen presenting cells (APC) such as macrophages or dendritic cells take up antigen from the graft, process and present it in the context of host MHC class II molecules. The APCs subsequently migrate to the lymphatic tissue (lymph nodes or tertiary lymphatic organs, known as TLOs(43) where they meet complementary T-cells(27). This interaction leads to activation of alloreactive T-cell continued by proliferation with subsequent activation of B-cells and migration that induce antibody formation. Activated alloreactive T-cells can then migrate to the area of the graft through blood flow. Recipient alloreactive T cells could be activated via either the indirect pathway (donor-derived peptides presented in the context of host MHC molecules) or the semidirect pathway (donor MHC class I molecules from fibroblasts or keratinocytes displayed on recipient APCs)(27)

Table 1

Grafting pattern and rejection

Animal	SLA-type	1st surgery*	Rejection (day)	2nd surgery*	Rejection (day)
20668	CC	2 DD-SC	8	2 DD-SC+2 CC-SC	<4
20838	CC	2 DD-SC	8	2 DD-SC+2 CC-SC	<4
20839	CC	2 DD-SC	8	2 DD-SC+2 CC-SC	<4
20863	CC	2 DD-SC	8	2 DD-SC+1 CC-SC+1 CC-STSG	<4 6 (STSG)
20866	CC	2 DD-SC	10	2 DD-SC+2 CC-SC	<4
21259	CC	2 DD-STSG	6	2 DD-STSG+2 CC-STSG	<4

* Animals received 2 autologous STSGs at each surgery with the exception of 20863. Animal 20863 received one autologous and one minor mismatched STSG (from animal #20839) at the 2nd surgery. Engraftment and course of rejection were compared to that of the minor mismatched SLA^{CC} construct (Figure S4).