StrataGraft Skin Substitute Is Well-tolerated and Is Not Acutely Immunogenic in Patients With Traumatic Wounds

Results From a Prospective, Randomized, Controlled Dose Escalation Trial

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Objective: The goal of this study was to assess the immunogenicity and antigenicity of StrataGraft skin tissue in a randomized phase I/II clinical trial for the temporary management of full-thickness skin loss.

Background: StrataGraft skin tissue consists of a dermal equivalent containing human dermal fibroblasts and a fully stratified, biologically active epidermis derived from Near-diploid Immortalized Keratinocyte S (NIKS) cells, a pathogen-free, long-lived, consistent, human keratinocyte progenitor.

Methods: Traumatic skin wounds often require temporary allograft coverage to stabilize the wound bed until autografting is possible. StrataGraft and cadaveric allograft were placed side by side on 15 patients with full-thickness skin defects for 1 week before autografting. Allografts were removed from the wound bed and examined for allogeneic immune responses. Immunohistochemistry and indirect immunofluorescence were used to assess tissue structure and cellular composition of allografts. In vitro lymphocyte proliferation assays, chromium-release assays, and development of antibodies were used to examine allogeneic responses.

Results: One week after patient exposure to allografts, there were no differences in the numbers of T or B lymphocytes or Langerhans cells present in StrataGraft skin substitute compared to cadaver allograft, the standard of care. Importantly, exposure to StrataGraft skin substitute did not induce the proliferation of patient peripheral blood mononuclear cells to NIKS keratinocytes or enhance cell-mediated lysis of NIKS keratinocytes in vitro. Similarly, no evidence of antibody generation targeted to the NIKS keratinocytes was seen.

Conclusions: These findings indicate that StrataGraft tissue is well-tolerated and not acutely immunogenic in patients with traumatic skin wounds. Notably, exposure to StrataGraft did not increase patient sensitivity toward or elicit immune responses against the NIKS keratinocytes. We envision that this novel skin tissue technology will be widely used to facilitate the healing of traumatic cutaneous wounds.

This study was registered at www.clinicaltrials.gov (NCT00618839).

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All authors contributed to the manuscript preparation and gave final approval of the manuscript. Additional responsibilities included the following: J.M.C. contributed to the trial design and was responsible for its oversight, functioned as the clinical liaison, assisted with sample collection, data compilation, and data interpretation. J.A.S. was responsible for the experimental design, conduct, and interpretation of the in vitro data. A.W. was responsible for the management and capture of clinical data. J.A.H. was responsible for the design, conduct and analysis of the *in vitro* proliferation and cytotoxicity data. C.A.R. was responsible for the conduct and capture of the clinical immunofluorescent data and contributed to the manuscript preparation. M.A.L. was responsible for data analysis and interpretation and manuscript preparation. M.J.S. was the lead clinical investigator who contributed to the trial design, patient enrollment, patient treatment, sample collection, and data interpretation. K.N.F. was responsible for enrolling and treating patients, sample collection, and data acquisition. L.D.F. was responsible for enrolling and treating patients, sample collection and data acquisition. D.M.C. was responsible for enrolling and treating patients, sample collection and data acquisition. A.R.C. was Principal Investigator on the NIH SBIR project that partially funded the clinical trial. As such, Dr Comer was involved in the design of the clinical study, and analysis and interpretation of clinical data from the study. Dr Comer was responsible for interactions with the data and safety monitoring board for this study. B.L.A.-H. was responsible for the overall design and management of the clinical study.

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Large traumatic cutaneous wounds often result in a life-threatening disruption of barrier function, which necessitates rapid wound closure to prevent dehydration and bacterial infection. Continuous renewal of the epidermis by the interfollicular keratinocyte progenitor cells of the basal layer is crucial for maintenance of the biochemical and mechanical properties of the skin. On the basis of the pioneering work of Rheinwald and Green,[1] methods have been developed to expand keratinocytes in culture for use in the treatment of severe burns. However, an inherent limitation of the in vitro culturing of human keratinocytes is the eventual depletion of the progenitor cell population by serial cultivation.[2] A consistent source of human epidermal progenitor cells would enable the development of effective treatments for a variety of cutaneous disorders. The previously described NIKS skin cell line is a long-lived, genetically stable, nontumorigenic, and pathogen-free human keratinocyte progenitor and is a reliable source as a universal donor.[3]

In recent years, numerous advances have been made in organotypic culturing systems to generate living bioengineered skin substitutes. By inducing terminal differentiation, these systems are capable

of recapitulating many of the structural and functional characteristics of the interfollicular epidermis. Among other features, these tissues form a basement membrane, develop stratified layers that supply protective barrier function, produce growth factors, and secrete host defense peptides.[4–7] Despite their therapeutic potential, bioengineered allogeneic tissues have the potential complication of antigenic stimulation of the host immune system. Keratinocytes, the predominant cell type in skin,[8,9] have historically been considered a passive cellular constituent. Human keratinocytes are classified as "nonprofessional" antigen presenting cells (APC)[10–12] because, although they express major histocompatibility complex (MHC) class I antigens on their cell surface, they lack constitutive expression of MHC class II antigens.[13,14] The most widely expressed MHC class II antigen, human leukocyte antigen (HLA)-DR, and the class I antigens HLA-A, -B, and -C are associated with tissue rejection in humans,[15,16] and therapeutic outcomes are dramatically improved when these molecules are closely matched between transplant donor and recipient. Within the healthy epidermis, the Langerhans cell is often considered the sole cell type capable of expressing MHC class II and efficiently presenting antigen.[17–20] Therefore, engineered skin substitute tissues that do not contain professional APC, such as Langerhans cells (LC) or leukocytes, greatly reduce the possibility of allograft antigen presentation.

StrataGraft human skin substitute, produced in vitro by the organotypic culture of NIKS keratinocytes, generates a stratified epidermal layer closely resembling that seen in intact human skin. The morphology, differentiation marker expression, basement membrane development, barrier function, and MHC expression of StrataGraft tissue were similar to native human skin, and to tissues generated in vitro from normal human epidermal keratinocytes (NHEK). StrataGraft was evaluated in a randomized, phase I/II clinical trial as a temporary coverage for patients with full-thickness skin loss of 5% or more total body surface area (TBSA). In this study, the immunogenicity of StrataGraft skin substitute was examined during an open-label, controlled, randomized, comparative, dose escalation study of the surgical management of complex skin defects in patients undergoing sequential skin reconstruction procedures. The immunological properties of this biological skin substitute were investigated before and after placement in a patient wound. Patient sensitization to the NIKS keratinocytes was also assessed before and after placement of StrataGraft tissue by examining the ability of NIKS cells to induce patient lymphocyte proliferation, the susceptibility of NIKS cells to patient natural killer (NK) cell-mediated lysis, and the development of NIKS-specific antibody responses. Further evaluation included histological examination of the allograft tissues after placement in the wound bed for 1 week. The results of this study indicate that StrataGraft tissue generated from NIKS keratinocytes has characteristics comparable to skin substitutes prepared from primary NHEK. Furthermore, StrataGraft skin substitute does not induce acute inflammatory responses in patients with full-thickness skin loss. Clinical assessment of the StrataGraft-treated wound beds indicated that StrataGraft skin tissue offers an innovative therapeutic solution for acute skin defects and will likely continue to function beyond the time frame investigated.[21] Phase II proof of concept studies investigating longer exposure to StrataGraft are warranted given the safety results obtained during this study.

METHODS

Monolayer and Organotypic Culture

Human neonatal foreskin tissue was obtained by informed consent in accordance with both Meriter Hospital (Madison, WI) and the University of Wisconsin–Madison institutional review boards. A description of the cell culture and tissue production is available online (Supplemental Digital Content 1, available at: http://links.lww.com/SLA/A115).

Study Enrollment

A phase I/II, multicenter, open-label, randomized, safety, and dose escalation trial was conducted from 2007 to 2008 under a clinical protocol on file with Center for Biologics Evaluation and Research at the Food and Drug Administration. The study was performed to assess autograft take and infection in 15 patients with full-thickness skin defects, who were intended to undergo sequential surgical procedures involving surgical skin debridement and temporary cadaver allograft placement followed by autografting.[21] The trial was conducted at the University of Wisconsin Hospital and Clinics (Madison, WI) and the Arizona Burn Center at Maricopa Medical Center (Phoenix, AZ) and funded in part by the National Institutes of Health. The patients enrolled in this study had wounds resulting from thermal burns ($n = 11$), surgical resection of soft tissue infection ($n = 2$), electrical burns ($n = 1$), and traumatic injury ($n = 1$). Mean patient age was 43.4 $\pm$ 14.7 years; 73.3% of the patients were male, and the mean full-thickness skin loss was 22.2% $\pm$ 18.0 TBSA.

Declaration of Helsinki, Informed Consent, and Health Insurance Portability and Accountability Act Statement

This trial was conducted in accordance with good clinical practices and Food and Drug Administration regulations and guidelines. These regulations and guidelines encompass all principles established by the Declaration of Helsinki and all its subsequent amendments. The research protocol and informed consent forms were approved by the respective institutional review board for each participating study site. The consent form was available in English and Spanish as needed. At the time of enrollment, all patients were assigned a sequential, de-identified number and clinical study site identifier. For the purposes of maintaining patient anonymity and to eliminate bias, all patient samples and representative photos were labeled with the coded patient number and the blinded wound site designation.

Patient Treatment and Study Design

Treatment for each wound site was randomized such that one half of a contiguous wound site received StrataGraft skin substitute and the other adjacent half received the standard of care, banked, cryopreserved, and cadaver allograft.[21] The allogeneic skin treatments were applied to the debrided wound site. Allografts were placed and secured with staples, photographed, and covered with nonadherent petrolatum impregnated gauze. Wound dressings were changed approximately every 2 days until allograft removal. Assessments were performed during each dressing change for allograft adherence, color, and visual signs of infection. One week after placement, the StrataGraft and cadaver allografts were removed. Subsequent autograft placement occurred when the wound bed was judged by the physician to be suitable to accept an autograft. Autografts were likewise covered with nonadherent petrolatum-impregnated gauze and dressings were changed at days 3, 6, and then daily until healed. Autografts were monitored for adherence, color, autograft take, and visual signs of infection. As additional patients were enrolled, the targeted dose of StrataGraft tissue was sequentially increased. The first 5 patients were treated with no more than 0.5% TBSA of StrataGraft skin substitute. The second cohort of 5 patients was treated with up to 1.0% TBSA of StrataGraft tissue. The third patient cohort was treated with no more than 1.5% TBSA of StrataGraft skin substitute.

Clinical and Laboratory Tests

Clinical and laboratory tests for the clinical trial patients were performed as described.[21]

Study Endpoints

The primary safety and efficacy outcome was the percentage of autograft take, assessed by the study team, 2 weeks after autograft placement. Additional safety and efficacy assessments included immunohistochemical (IHC) and indirect immunofluorescent evaluations of allograft tissues after their removal from the wound site. Systemic immune responses were evaluated using panel reactive antibody (PRA) assessment, mixed leukocyte reactions, and ^{51}Chromium-release cytotoxicity assays.

Histology

The histologic preparation and staining procedures of the tissue samples were performed using standard methods and are described in detail online (Supplemental Digital Content 1, available at: http://links.lww.com/SLA/A115).

Peripheral Blood Mononuclear Cells Functional Assays

Peripheral blood mononuclear cells (PBMC) purification and cellular activation assays were performed by standard methods and a complete description can be found online (Supplemental Digital Content 1, available at: http://links.lww.com/SLA/A115).

RESULTS

NIKS Keratinocytes Produce a Morphologically Accurate Stratified Squamous Epithelial Tissue

Using histology and indirect immunofluorescence (IIF), the structure and differentiation state of tissues generated from primary NHEK or from NIKS keratinocytes were examined and compared to native human skin tissue. Hematoxylin and eosin (H&E) staining revealed that the epidermal morphology of tissues made from NIKS cells was indistinguishable from that of tissue prepared with NHEK (Fig. 1A). All tissues contained a distinct basal layer consisting of cuboidal cells with a high nucleus-to-cytoplasm ratio. Cells within the spinous layer exhibited flattening typical of terminal epidermal differentiation. Distinct staining of keratohyalin granules within the granular layer was evident in all tissues, as was a well-defined enucleated stratum corneum, both characteristic of mature epidermis. Each of these histological features was also present in native skin that typically exhibits a thinner stratum corneum as a result of friction and sloughing.

The ability of NIKS and NHEK organotypic cultures to generate a functional barrier was assessed by measuring skin surface electrical impedance. The rate of surface electrical impedance change correlates with the rate of transepidermal water loss, indicating the integrity of the epidermal permeability barrier.[22,23] Both NIKS and NHEK produced tissue with barrier function that was comparable to intact human forearm skin (Fig. 1B). In contrast, loss of barrier function was readily detectable using surface electrical impedance after tape stripping of human forearm skin.

Markers of keratinocyte differentiation were visualized by IIF to evaluate cellular differentiation and tissue morphogenesis. Staining for the membrane-bound intermediate-stage differentiation marker type I transglutaminase was seen in the suprabasal layers of both NIKS and NHEK tissues, continuing up to the stratum corneum (Fig. 1C). Likewise, transglutaminase staining in native foreskin tissue was membrane-bound and located only within the mature epidermal layers. Expression of filaggrin, a marker of intermediate-to-late stage keratinocyte differentiation, was appropriately contained within the granular layer of both NIKS and NHEK tissues, similar to that seen in native skin. Additional markers of stratified squamous epithelia differentiation including involucrin, structural proteins keratin 1, keratin 14, and keratin 2, as well as the cell adhesion proteins E- and P-cadherin, were assessed and exhibited appropriate localization in NIKS and NHEK tissues (data not shown).

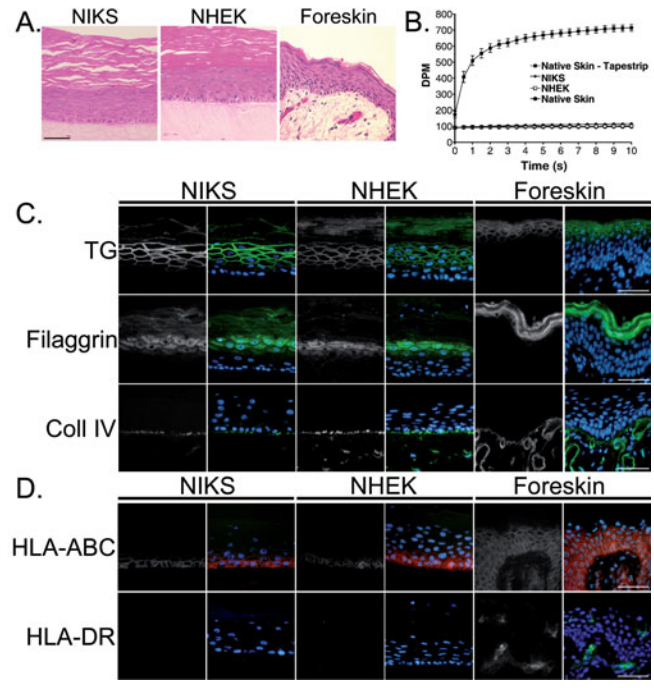

FIGURE 1. In vitro–generated tissues have characteristics comparable with those of native skin. (**A**) H&E stained sections from NIKS and NHEK skin substitutes, and human neonatal foreskin tissue, exhibit typical epidermal morphology, containing a single layer of basal cells, beneath stratified spinous, granular, and cornified layers. Scale bar = 50 μm. (**B**) Barrier function was measured using a Nova DermaPhase meter (DPM), which measures skin surface electrical impedance in arbitrary units. DPM values reflect electrical impedence and are inversely related to surface hydration; therefore, lower DPM values indicate a dryer surface. Tissues made from NIKS (StrataGraft) or NHEK exhibit barrier function comparable with that of native human skin. The values obtained for intact skin and skin where the barrier function has been disrupted by tape stripping are presented as controls. Data represent mean ± SEM. (**C**) Antibodies specific to type I transglutaminase (TG), filaggrin, and collagen IV (Coll IV) were used to examine the differentiation pattern present in NIKS and NHEK tissues and human neonatal foreskin. Grey scale images for each protein examined are shown. Color-merged images display sections counterstained with Hoechst 33,258 to visualize nuclei (blue). Scale bars = 50 μm. (D) Expression of MHC antigens was also assessed (grey scale), with color-merged images depicting HLA-ABC in red, HLA-DR in green, and Hoechst 33,258 in blue. Scale bars = 50 μm.

Collagen IV was detected by IIF to investigate basement membrane formation in tissues generated in vitro (Fig. 1C). All tissues showed appropriate deposition of collagen IV at the epidermal-dermal junction. Staining was varied throughout the length of the tissue, revealing both focal and diffuse staining patterns. In foreskin tissue, collagen IV staining was present at the epidermal-dermal junction with additional localization to the interface of resident hair follicles

in the dermis. These data demonstrate that NIKS keratinocytes terminally differentiate in organotypic culture to form a mature, fully differentiated, and biologically functional tissue that possesses barrier function comparable with that of intact human skin.

NIKS and Primary Keratinocytes Express Similar Levels of Immunological Cell Markers

To determine the baseline immunophenotypic characteristics of NIKS tissue, the expression of several cell surface molecules that are involved in immune responses was examined. Immunofluorescence was used to detect the MHC class I human leukocyte antigen complex (HLA-ABC), the most abundant class II antigen (HLA-DR), and the costimulatory molecules B7-1, B7-2, and CD40. Positive staining for HLA-ABC was detected in the basal and immediately suprabasal layers of NIKS and NHEK tissues (Fig. 1D). In native neonatal foreskin tissue, expression of HLA-ABC antigen was present in basal and additional suprabasal layers. HLA-ABC staining was appropriately localized to the cell membrane in all tissues.

Keratinocytes do not constitutively express MHC class II or costimulatory molecules on their surface and are therefore categorized as nonprofessional APC. As expected, both NIKS and NHEK tissues were negative for HLA-DR antigen staining (Fig. 1D). In contrast, HLA-DR expression was apparent in both the dermal and epidermal compartments of native foreskin, with cellular morphology indicative of dendritic cell or leukocytic expression and not keratinocyte expression. Expression of the costimulatory molecules B7-1, B7-2, and CD40 was not detectable in NIKS, NHEK, or native foreskin tissues (data not shown). These data suggest that although NIKS cells will likely be recognized as allogeneic by the immunocompetent host through the expression of class I antigens, they lack any constitutive antigen presentation capability.

StrataGraft Human Skin Substitute Maintained Normal Tissue Architecture and Remained Viable After 1 Week in the Patient Wound Bed

StrataGraft skin substitute, which is produced using NIKS keratinocytes, was compared to cryopreserved cadaver allograft, the standard of care, in the conditioning of debrided, full-thickness wounds before autograft placement. A phase I/II, randomized, controlled, safety and dose escalation trial was conducted in 15 patients whose wounds before autografting were temporarily covered with StrataGraft and cadaver allografts in a randomized, split-wound format. The primary safety and efficacy outcomes of this trial were recently published[21] and demonstrate that StrataGraft was well-tolerated and comparable to the standard of care in the surgical management of large full-thickness skin defects. No evidence of product related adverse events was seen. Figure 2 summarizes the study design and major evaluation points and immunological assessments. Allografts were removed from the wound beds 1 week after placement and samples were fixed, sectioned, and stained to assess tissue structure, composition, and proliferative capacity. Histological analysis of both StrataGraft and cadaver allografts confirmed that the tissue morphology and stratification remained intact during the 1-week placement period (Fig. 3). Specifically, a single basal layer of cuboidal cells was observed in both StrataGraft and cadaver allograft tissue. Likewise, the spinous and granular layers, and the stratum corneum, were evident in both StrataGraft and cadaver allograft tissues after removal from the wounds.

Immunohistochemical staining for Ki67, a protein expressed only in proliferating cells,[24] was used as an indicator of allograft viability. StrataGraft samples consistently exhibited Ki67 staining in both basal and suprabasal keratinocytes (Fig. 3B), as has been shown previously in native human skin during wound healing.[25] Cadaver

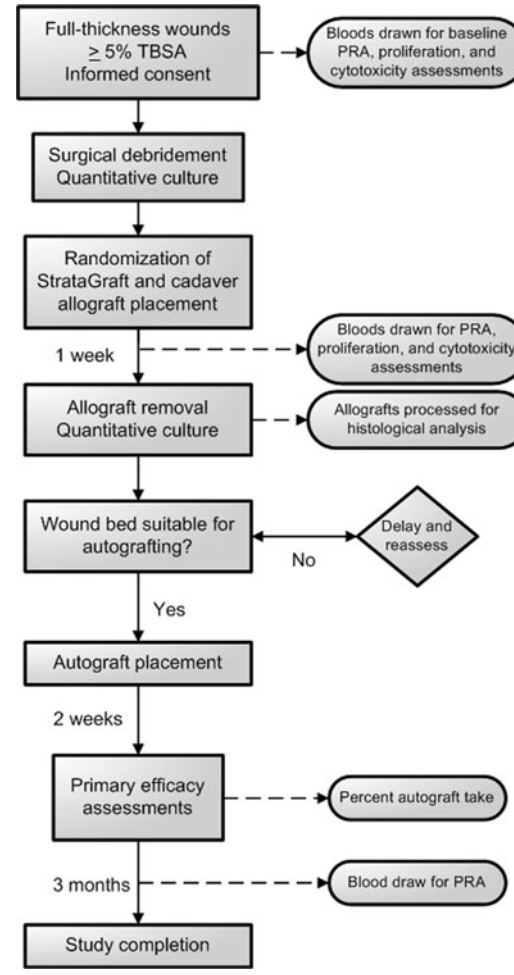

FIGURE 2. Overview of clinical trial design. Fifteen patients with full-thickness skin defects of ≥5% total body surface area were surgically debrided and wound beds were covered with StrataGraft and cadaver allografts placed adjacent to each other. Allografts were removed after 1 week and processed for histologic analysis. Patients were autografted when the wound beds were deemed by the clinician to be suitable for autografting. The primary efficacy endpoint was the percent of autograft take 2 weeks after autograft placement. A panel of secondary safety and efficacy analyses were performed at baseline, 1 week after allograft placement, and after 3 months.

allograft samples exhibited Ki67 staining almost exclusively in the basal cell layer, although the percentage of positive cells was highly variable (Fig. 3B). Two blinded observers each scored at least 400 cells from each sample as either positive or negative for Ki67 staining and the ratio of stained-to-total cells was reported as the proliferation index (PI). Intensity of staining was not considered in this evaluation. Although not statistically significant using a paired, 2-tailed Student t test and a 95% confidence interval ($P = 0.110$), the mean PI of StrataGraft tissue was higher (0.521) than that of cryopreserved cadaver allograft (0.288). The median value for StrataGraft skin tissue was 0.567 as compared with 0.189 for cadaver skin (Fig. 3C). Furthermore, the range of values for StrataGraft (0.523) was approximately half that for cadaver skin (0.944), suggesting that StrataGraft is a

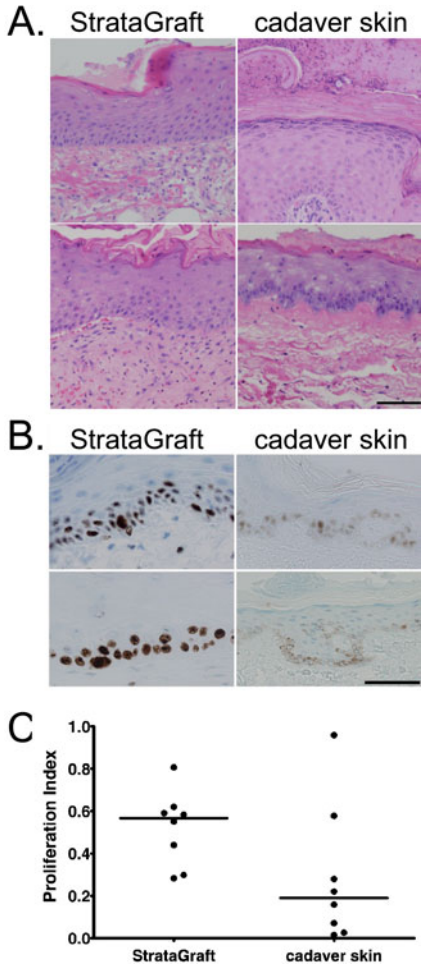

FIGURE 3. StrataGraft skin substitute remains intact and viable after placement in the wound bed. (**A**) Representative paraffin-embedded H&E-stained sections of StrataGraft and cadaver allografts after removal from the wound bed of 2 different patients. Scale bar = 50 μm. (**B**). Representative images of StrataGraft and cadaver allograft samples stained for Ki67 expression after removal from the wound beds of 2 different patients reveal Ki67 staining of viable cells present in basal and suprabasal layers (brown). Scale bar = 50 μm. (**C**) Sections were assessed by 2 blinded observers and basal keratinocytes were scored as positive or negative for Ki67 staining. At least 400 cells from each sample were counted and the proliferation index (ratio of positive cells to the total cells counted) was determined. Shown are paired StrataGraft and cadaver allograft sample PI values from 8 individual patients. The bar indicates the median value. McNemar's test, $P = 0.13$.

more consistent source of viable and biologically active cells for the conditioning and coverage of cutaneous wounds.

StrataGraft Skin Tissue Did Not Induce Acute Inflammatory Infiltrate In Vivo

Infiltration of T lymphocytes, B lymphocytes, and LC into StrataGraft and cadaver allografts was assessed as an indicator of allograft antigenicity and early graft rejection. Samples were assessed for IHC staining of the T-cell marker CD3. Modest numbers of T cells were found in either the epidermal or dermal compartments of the allografts (Fig. 4). Scoring by 2 independent blinded observers revealed no statistically significant differences in the numbers of CD3 positive cells in the dermis and epidermis of StrataGraft skin substitute compared to cadaver allograft. Staining for the B-cell marker CD20 revealed few, if any, CD20 positive cells in and around the epidermis of either allograft (Fig. 4). Occasional samples contained significant B-cell infiltrate in the wound bed material adjacent to the allografts; however, no statistically significant differences between StrataGraft and cadaver allografts were seen. The presence of LC in the epidermis of StrataGraft and cadaver allografts was assessed by IHC staining of CD1a. CD1a positive cells were observed in both StrataGraft and cadaver allograft (Fig. 4); however, no statistically significant differences in cell numbers were detected. Taken together, these data show that after placement of StrataGraft skin substitute for 1 week in full-thickness wounds, normal tissue architecture is maintained with no substantial infiltration of lymphocytes. These data suggest that StrataGraft skin substitute does not induce an acute inflammatory response in patients with traumatic skin loss.

MHC Class I But Not Class II Expression Was Detected in Epidermal Layers After Placement in Full-thickness Wounds

After placement in full-thickness wounds for 1 week, StrataGraft and cadaver allografts were assessed for HLA-ABC and HLA-DR staining as indicators of early graft rejection, inflammation, tissue antigenicity, and epidermal reactivity. Evaluation was based on the presence and localization of staining, rather than intensity. HLA-ABC expression within StrataGraft and cadaver allografts was membrane-localized and present in the majority of stratified keratinocyte layers (Fig. 5A). This staining pattern is unlike the expression seen in ungrafted tissues, which was restricted to the basal and immediately suprabasal layers (Fig. 1D). Few HLA-DR positive cells were observed within the epidermis of either StrataGraft or cadaver allografts

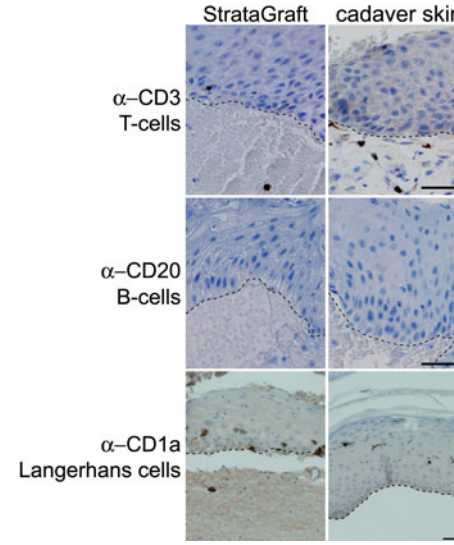

FIGURE 4. StrataGraft skin substitute does not exhibit acute inflammatory infiltrates. Representative images of StrataGraft and cadaver allografts removed from the wound beds of 2 different patients, stained (brown) for CD3 to indicate T-cell infiltrates, CD20 as a marker of B lymphocytes, and CD1α to identify Langerhans cells. Scale bars = 100 μm.

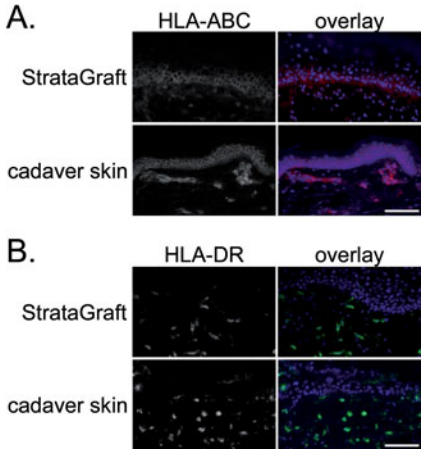

FIGURE 5. HLA-ABC, but not HLA-DR, is expressed in the basal and suprabasal epidermal keratinocytes of both StrataGraft and cadaver allografts after placement in the wound bed. The expression and localization of HLA-ABC and HLA-DR were assessed by immunofluorescence. Shown are representative grey scale and color images of postplacement StrataGraft and cadaver skin allografts stained for HLA-ABC (A; shown in red) and HLA-DR (B; shown in green) after removal from the wound beds of 2 different patients. Nuclei were counterstained with Hoechst 33,258 (blue). Scale bar = 100 μm.

(Fig. 5B). Cells that expressed HLA-DR were found predominantly within the dermis with little infiltration into the epidermal portion of the allografts. The morphology of the few HLA-DR positive cells within the epidermis was suggestive of dendritic cells and not of keratinocytes. Localization of HLA-DR staining was similar between StrataGraft tissue and cadaver skin. These data indicate that the allogeneic keratinocytes of StrataGraft or cadaver skin did not exhibit detectable expression of HLA-DR after 1 week in the wound bed as might be seen during an acute inflammatory response.

Tissues Generated From NIKS and Primary NHEK Regulate Immunological Markers Similarly in Response to γ-Interferon (IFN-γ) Stimulation In Vitro

Previous work in vitro has shown that NHEK express HLA-DR and CD40 upon exposure to the proinflammatory cytokine IFN-γ.[11,26–34] However, even after IFN-γ treatment, primary keratinocytes do not express the costimulatory molecules B7-1 or B7-2 at levels sufficient to induce T-cell proliferation.[26,27] To confirm that the lack of inflammatory infiltrate seen in the clinical samples was not the result of a defect in the cellular response to stimulation, immunofluorescence was used to determine whether StrataGraft upregulated these immunological markers in response to cytokine stimulation. Tissues were treated in vitro with 50, 100, or 1000 units/mL of recombinant human IFN-γ (rhIFN-γ) for 5 days. StrataGraft showed enhanced membranous expression of the MHC class I antigens, HLA-ABC, after IFN-γ exposure (see Fig., Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A116). At all IFN-γ doses, HLA-ABC was detected in the majority of the stratified keratinocyte layers whereas the vehicle control tissues exhibited HLA-ABC expression that was restricted to the basal and immediately suprabasal layers.

After treatment with rhIFN-γ, HLA-DR expression was detectable in the cell membranes of both basal and immediately suprabasal NIKS cells (see Fig., Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A116). Moreover, there was a dose dependent increase in the number of tissue layers expressing HLA-DR antigen. IFN-γ-treatment of NIKS tissues also induced a dose-dependent increase in the expression of CD40, although expression was restricted to the basal layer even at the highest dose of IFN-γ (see Fig., Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A116). NIKS tissues did not exhibit detectable levels of B7-1 or B7-2 even when exposed to high levels of rhIFN-γ (1000 U/mL, see Fig., Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A116). NHEK tissues showed a similar dose-dependent increase of HLA-ABC, HLA-DR, and CD40 expression with no detectable B7-1 or B7-2 expression after exposure to rhIFN-γ (data not shown). No changes in localization or staining intensity of the keratinocyte differentiation markers transglutaminase, filaggrin, involucrin, E- and P-cadherin, keratins 1, 14, and 2 were observed in IFN-γ–treated NIKS or NHEK tissues when compared to vehicle controls (Supplemental Digital Content 3 and data not shown, available at: http://links.lww.com/SLA/A117). These data indicate that NIKS keratinocytes are capable of expressing MHC class II on their surface. However, NIKS keratinocytes do not express detectable B7-1 or B7-2 after IFN-γ stimulation, suggesting that they would be unlikely to act as APC and activate acute T lymphocyte responses directly.

PBMC From Clinical Trial Patients Did Not Exhibit Increased Responsiveness Against NIKS Keratinocytes After Exposure to StrataGraft Tissue

To determine whether exposure to StrataGraft tissue increased the numbers of patient white blood cells specific for NIKS keratinocytes, in vitro cellular proliferation assays were performed. Patient PBMC were assessed in a cellular proliferation assay both at baseline (pretreatment) and again at the time of allograft removal (posttreatment). Proliferation assays were carried out for 3 and 6 days and the stimulation index (stimulation index [SI] = counts per treatment/counts in media control) calculated. The mean baseline value for patient PBMC cultured with medium for 6 days was 1738 $\pm$ 2906 counts (SI = 1). There was a significant increase in the mean value for baseline patient PBMC stimulated with the positive control phytohemagglutinin (PHA) (SI = 22.8; $P < 0.0001$). Baseline PBMC did not proliferate in response to irradiated autologous PBMC (SI = 1.13). As expected, there was a reproducible increase in counts to irradiated allogeneic PBMC (SI = 9.05; $P < 0.0001$); however, no increase in proliferation was seen with the irradiated NIKS cells at 1×10^5 (SI = 0.266) or 1×10^4 (SI = 0.395) cells per well.

Mean sample values for patient PBMC obtained after allograft removal were similar to those with PBMC obtained at baseline. Responses to PHA and irradiated allogeneic PBMC after allograft placement (SI = 10.05 and SI = 6.21, respectively) were comparable with those seen at baseline ($P = 0.21$ and $P = 1.00$, respectively). The mean responses of patient PBMC to irradiated NIKS cells before and after treatment were not statistically significant (SI = 0.225 and 0.492, respectively; $P = 0.80$). The patient-specific SI values for PBMC cocultured with irradiated NIKS cells are shown in Table 1 and indicate a lack of allogeneic sensitization against the NIKS keratinocytes. There were no significant changes between the baseline and posttreatment responses in the 3- or 6-day assays (Table 1 and data not shown). Likewise, there were no differences in any values obtained from the 3 patient cohorts.

To test whether the lack of PBMC responsiveness was due to reduced immunocompetency in the patient population, in vitro

TABLE 1. Patient PBMC Proliferation in Response to NIKS Cells

		Proliferation Assay (Stimulation Index*)								
		10^4 NIKS Cells				10^5 NIKS Cells				
		Pretreatment		Posttreatment		Pretreatment		Posttreatment		
Patient Number		3 day	6 day	3 day	6 day	3 day	6 day	3 day	6 day	(+/−)
Cohort 1: up to 0.5% TBSA	1	6	1	9	3	6	0	6	3	(−)
	2	7	4	2	0	11	5	5	0	(−)
	3	0	0	1	0	0	0	1	0	(−)
	4	1	1	2	3	1	1	1	3	(−)
	5	9	1	1	1	7	0	4	0	(−)
Cohort 2: up to 1.0% TBSA	6	2	0	9	9	15	2	10	8	(−)†
	7	6	4	9	5	5	2	36	10	(−)†
	8	1	0	10	1	5	1	9	2	(−)†
	9	1	0	1	0	1	0	1	0	(−)
	10	2	5	1	1	2	4	0	1	(−)
Cohort 3: up to 1.5% TBSA	11	1	1	1	1	1	0	1	1	(−)
	12	2	2	1	1	2	0	1	0	(−)
	13	1	4	7	2	1	1	3	1	(−)†
	14	1	1	2	1	3	1	1	1	(−)
	15	2	0	1	0	1	0	0	0	(−)

*Stimulation index = counts per treatment/counts in the media.

†Very low level ^{3}H-TdR incorporation in background and NIKS samples. All counts are <3500 and thus the changes in the stimulation indices are not considered biologically significant or indicative of in vivo sensitization.

(+) indicates a positive proliferation response; (−), a negative proliferation response.

cellular proliferation assays were performed to determine whether allogeneic NIKS keratinocytes induced the proliferation of PBMC from healthy individuals. Peripheral blood mononuclear cells isolated from healthy, immunocompetent donors were cocultured with irradiated NIKS keratinocytes. PBMC proliferation was not stimulated by irradiated allogeneic NIKS cells (see Table, Supplemental Digital Content 4, available at: http://links.lww.com/SLA/A118). To account for any inhibitors of proliferation that may be produced by the NIKS cells, serial 10-fold dilutions of NIKS keratinocytes from 1×10^5 to 1×10^2 cells per well were also tested with similar results (see Table, Supplemental Digital Content 4, and data not shown, available at: http://links.lww.com/SLA/A118). Comparable data were obtained with PBMC from a total of 4 human volunteers over 10 replicate experiments. These in vitro proliferation data suggest that the placement of StrataGraft skin tissue in a wound bed does not enhance patient PBMC activation toward NIKS or allogeneic PBMC. Furthermore, these data indicate that patient PBMC obtained after StrataGraft tissue placement do not have an altered proliferative response compared with that before allograft placement. Taken together, these data suggest that NIKS keratinocytes do not induce the activation of PBMC directly.

Patient PBMC Exhibited Minimal NK Cell-Mediated Killing of NIKS Cells In Vitro

Patient PBMC obtained at study entry (pretreatment) or at the time of allograft removal (posttreatment) were tested in an in vitro cytotoxicity assay to assess the level of NK cell activity toward NIKS keratinocytes (Table 2) and the NK-sensitive cell line K562. Patient PBMC mediated only low-level cytolytic activity against the K562 targets at all effector-to-target (E:T) ratios tested and there was no significant difference in the cytotoxicity seen with PBMC obtained at baseline as compared with those obtained after allograft exposure. At the E:T ratio of 50:1, mean patient cytotoxicities toward K562 cells were 13.10% ± 9.48 pretreatment and 11.09% ± 10.15 posttreatment (n = 13). NIKS keratinocytes used as target cells were relatively NK cell-resistant and patient PBMC obtained before StrataGraft placement exhibited little lytic activity toward them at all E:T ratios tested (mean patient cytotoxicity 7.68% ± 9.58 for 50:1 E:T; n = 14). To ensure that the apparent resistance of NIKS cells to NK cell killing was not the result of a potentially immunocompromised patient population, the spontaneous lytic activity of NK effector cells against NIKS keratinocytes was assessed. Peripheral blood mononuclear cells from healthy donors were cultured with ^{51}Cr-labeled target cells: either the allogeneic NIKS keratinocytes or the positive control, NK cell-sensitive, MHC class I^{low}, leukemia cell line K562. Quadruplicate assays were performed on PBMC from 4 human volunteers in more than 10 replicate experiments with similar results. No cellular lysis resulted from coculturing the NIKS keratinocytes with healthy donor effector PBMC, suggesting that NIKS cells are inefficient NK-cell targets (see Table, Supplemental Digital Content 5, available at: http://links.lww.com/SLA/A119).

At the time of allograft removal, patient PBMC did not exhibit enhanced lytic activity at any E:T ratio when compared to baseline values (9.03% ± 12.26 for 50:1 E:T; n = 14). These in vitro cytotoxicity data suggest that the exposure of patients to StrataGraft skin substitute for 1 week did not induce in vivo priming of patient PBMC NK cell lytic activity against NIKS cells in vitro. Significantly, the PBMC obtained after allograft placement did not have an altered response from the PBMC obtained before allograft placement.

Clinical Trial Patients Did Not Develop Antibody Responses Targeted to the HLA Types of NIKS Cells

To assess the development and specificities of antibodies directed against MHC antigens by study participants, PRA levels were measured before allograft placement, at the time of allograft removal (~7 days), and again at the study completion (~90 days; Table 3). It is important to note that most of the patients received blood product transfusions during the study and all patients were exposed not

TABLE 2. Patient PBMC Cytotoxicity Against NIKS Cells

		Cytotoxicity Assay (% Cytotoxicity for 50:1 E:T)		
Patient Number		**Pretreatment**	**Posttreatment**	**(+/−)**
Cohort 1: up to 0.5% TBSA	1	4	5.1	(−)*
	2	11.9	5.5	(−)
	3	7.1	18.6	(−)*
	4	4	3.5	(−)
	5	31.6	45.6	(+/−)†
Cohort 2: up to 1.0% TBSA	6	3.2	8.7	(−)*
	7	8.8	24	(+/−)†
	8	25.1	6.1	(−)
	9	2.1	6.5	(−)
	10	−1.8	3.8	(−)
Cohort 3: up to 1.5% TBSA	11	3.1	2.8	(−)
	12	N/T	−1	(−)
	13	−1.9	−4.1	(−)
	14	3.6	3	(−)
	15	6.7	7.3	(−)

*All increases in percentage cytotoxicity values over baseline are less than 20% even at the 50:1 effector to target ratio for patients 1 and 3 and less than 10% for patient 6. Furthermore, the level of cytotoxicity does not decrease with the dilution of effector cells so these changes are not considered biologically significant or indicative of in vivo sensitization.

†Patients 5 and 7 had the highest pre sample cytotoxicity values compared to all patients entered in the trial. Their post-treatment values slightly increased from their pretreatment values. This increase may be within day-to-day variability of this assay but some level of in vivo sensitization cannot be ruled out.

N/T indicates not tested; (+), a positive cytotoxic response; (−), a negative cytotoxic response; (+/−), a positive response is not ruled out.

only to the StrataGraft skin substitute (0.3%–1.5%TBSA) but also to cryopreserved cadaveric allografts (4.4%–72.1% TBSA). Six of the 15 patients exhibited elevated PRA values for at least 1 time point; however, all of these patients exhibited reactivities to HLA antigens that are absent on both NIKS cells and the dermal fibroblasts of the StrataGraft tissue. Five of these 6 patients received blood product transfusions during the study, 1 patient had an infection in the wound bed at the time of allograft removal, and the full-thickness wounds in 2 of these patients were the result of necrotizing fasciitis. Patient 4 developed antibodies to a single HLA allele expressed by NIKS keratinocytes; however, it is unlikely that this reactivity was a targeted response to the StrataGraft skin substitute since that individual also developed antibody specificities against other HLA alleles not expressed in StrataGraft tissue and did not develop additional reactivities to other HLA antigens on the NIKS keratinocytes. It is also important to note that this individual not only had an elevated baseline PRA value but was also in the low-dose StrataGraft tissue group. These data indicate that StrataGraft human skin substitute is not acutely antigenic and does not elicit a potent antibody response.

DISCUSSION

We have recently reported data from this phase I/II randomized safety and dose escalation clinical trial showing that StrataGraft skin substitute is well tolerated and comparable to cadaver allograft in the temporary treatment of debrided wounds before autograft placement.[21] This living skin substitute, composed of the NIKS keratinocyte progenitor cell line, was equivalent to cadaver skin in terms of wound bed preparation and did not increase the incidence of wound infection in this patient population. These data, in conjunction with the data presented here, illustrate the clinical utility of StrataGraft skin substitute as a pathogen-free, universal source of human skin in the temporary management of full-thickness skin wounds of several etiologies. As a nontumorigenic source of long-lived, genetically identical keratinocytes, the use of the NIKS keratinocyte cell line is advantageous when compared with other primary keratinocyte samples that senesce after multiple passages. The work described here characterizing the antigenic properties of StrataGraft skin substitute before and after patient exposure supports the continued clinical development of this biologically active skin tissue. The major findings in this study are that StrataGraft skin substitute maintains normal tissue architecture, remains viable after 1 week in acute, full-thickness wounds, is well tolerated by recipients, and does not induce an acute immune response.

After removal from the patient wound bed, the epidermal stratification of the StrataGraft remained intact. A single layer of cuboidal basal cells with suprabasal spinous, granular, and cornified layers was evident in the tissues postplacement, suggesting a lack of acute immune response to and resultant tissue destruction of the allografts. In addition, keratinocytes within the basal compartment of the stratified tissue stained positively for the cell cycle protein Ki67. Expression of Ki67 has been used as an indicator of keratinocyte proliferation and increases during wound healing.[24,35] Normal proliferative indices (PI; stained cells-to-total cells) vary on the basis of numerous factors including the anatomical location of the skin and the age of the individual.[36,37] The data shown here indicate that the PI values for StrataGraft skin substitute are more uniform than those for cryopreserved cadaver allograft. These data suggest that StrataGraft is a more consistent source of viable keratinocytes that will respond reliably within the context of a wound bed.

Although they could be found in the wound bed, T cells were only occasionally seen in close apposition to the epidermis, further suggestive of a lack of antigenicity of the allograft. Limited evidence of B lymphocytes in or around the epidermal layers was observed. The significance of LC in the epidermis of both StrataGraft and cadaver allograft tissues after placement in the wound bed is unclear. Historically, LC have been thought to play a role in graft rejection because they express MHC class II and are potent APC.[38] However, recent work has suggested that LC may be important in the development of immunological tolerance.[39] While the exact role that LC play during transplantation may still be debated, it is clear that these cells traffic rapidly into and out of the skin and their presence in StrataGraft skin substitute indicates that StrataGraft tissue readily allows cellular trafficking. In sum, there was no evidence of rapid infiltration of leukocytes including T and B lymphocytes nor was there evidence of rapid destruction of the allograft architecture that is seen when there is significant neutrophilic or mononuclear cell influx and activation seen during acute inflammatory responses. Over the years, many studies have focused on determining the immunogenic elements of allogeneic tissue responsible for acute graft rejection. Studies have shown that passenger leukocytes are largely responsible for this response[40,41] and that the rejection of transplanted tissues can take place either directly or indirectly. Acute rejection most often occurs as a result of direct allorecognition in which intact MHC class I and II molecules on the allografted tissue are recognized by the recipient T cells. This type of direct response tends to be quite rapid and the transplanted tissue is quickly recognized, infiltrated by recipient leukocytes, and broken down. Indirect allorecognition occurs when transplanted donor cells die and their MHC class I or II molecules are processed and presented on the recipient APC. This type of alloresponse tends to occur more slowly and has been shown to play a primary role in chronic tissue rejection and a lesser role in acute immune reactions.[42,43] Previous work has shown that the incubation of allogeneic human tissue in vitro before transplantation reduces its

TABLE 3. MHC Class I Panel Reactive Antibody Results

Cohort #	Patient Sex	Mode of Injury	% TBSA	% TBSA Treated	Day	Class I PRA%	Antibody Specificities Detected	Blood Product Transfused
1: up to 0.5% TBSA	1 Male	MVC degloving			0	0		
			5	0.5	7	N/T		
					90	0		
	2 Male	MVC-burn			0	0		PRC, FFP¶
			17.5	0.3	7	0		PRC, FFP||
					90	0		
	3 Female	Thermal burn			0	0		
			6	0.3	7	10*		
					90	46	B7,27 †B35,52,57,+	
	4 Male	Thermal burn			0	3		PRC¶
			16	0.3	7	28	B8,14,18,39+	PRC||
					90	36	B8,14,18,39,35,‡51+	
	5 Male	Thermal burn			0	0		
			13	0.3	7	0		
					90	0		
2: up to 1.0% TBSA	6 Female	Necrotizing Fasciitis			0	70	Multiple§	PRC^{G}
			8	0.6	7	90	Multiple§	PRC^{G}
					90	97	Multiple§	
	7 Female	Necrotizing Fasciitis			0	2		PRC, FFP, PLT||
			15	0.9	7	13	B7	PRC, FFP, PLT**
					90	0		
	8 Male	Explosion burn			0	10		PRC||
			73	0.9	7	7		PRC**
					90	2		PRC**
	9 Male	Explosion burn			0	0		
			12	0.9	7	0		
					90	0		
	10 male	Explosion burn			0	0		PRC¶
			25	0.6	7	0		PLT||
					90	0		
3: up to 1.5% TBSA	11 Female	Thermal burn			0	0		
			13	0.9	7	0		PRC||
					90	0		
	12 Male	Electrical burn			0	0		
			17.5	1.2	7	NT		
					90	0		
	13 Male	Explosion burn			0	0		PRC¶
			42.5	1.5	7	17	†B35,39	PRC||
					90	0		
	14 Male	Thermal burn			0	0		PRC, FFP||
			31	1.5	7	0		PRC, FFP||
					90	0		
	15 Male	Thermal burn			0	0		
			38.5	1.5	7	0		PRC||
					90	0		
Mean ± SD	73.3% Male		22.2 ± 18.0			10.0 ± 23.4		

*Patient had a wound bed infection at the time of allograft removal confirmed by quantitative culture at PTD 7.
†Weakly positive.
‡NIKS keratinocytes are HLA-B51.
§Too many reactive cells to reliably detect the antibody specificities.
¶Patient received a blood transfusion on the date of the blood draw for PRA.
||Patient received a blood transfusion several days before the date of the blood draw for PRA.
**Patient received a blood transfusion within a month before the date of the blood draw for PRA.
Day 0 indicates treatment application; day 7 ± 24 hours, removal of allografts; day 90 ± 1 week, study completion; FFP, fresh frozen plasma; MVC, motor vehicle collision; NT, not tested; PLT, platelets; PRC, packed red blood cells; PTD, post-treatment day.

immunogenicity. This is likely due to the death of passenger leukocytes during the culturing period, which reduces or eliminates acute allorecognition responses.[44–46] StrataGraft skin substitute does not contain leukocytes or dendritic cells and therefore does not generate a direct alloresponse in the recipient.

Human skin is constantly exposed to a tremendous number of environmental antigens and the presence of immunological markers on the keratinocyte surface is directly affected by the microenvironment in which these cells exist. Once a cutaneous wound has occurred, the milieu of proinflammatory cytokines may provoke keratinocytes to become active participants in the cutaneous immune response. The expression of MHC class I and class II antigens, in particular, HLA-A, -B, and -DR, has been closely associated with tissue rejection in humans.[15,16] The ability of keratinocytes to present antigen to $CD4^+$ T cells is tightly controlled through the regulated expression of HLA-DR and essential costimulatory molecules on their surface. Previous work has shown that the potent proinflammatory cytokine IFN-γ is able to induce HLA-DR expression in keratinocytes within a cultured skin substitute.[47] The data presented here indicate that both NIKS and primary NHEK tissues lack constitutive expression of the MHC class II antigen HLA-DR. In vitro exposure of the tissues to IFN-γ was sufficient to enhance the expression of HLA-ABC and induce the expression of HLA-DR and the costimulatory molecule, CD40, similar to that seen in other skin substitutes.[47] However, even at high concentrations of IFN-γ, expression of the key costimulatory molecules B7-1 or B7-2 was not detected.

In contrast to what one may anticipate during an acute inflammatory response and unlike that seen in animal models of skin grafting,[48,49] placement of StrataGraft skin substitute in acute full-thickness wounds in human patients is not sufficient to induce the expression of HLA-DR on the allografted keratinocytes. The HLA-DR expression seen in the tissues was instead suggestive of the presence of dendritic cells and leukocytes. This lack of HLA-DR expression by the keratinocytes coupled with the lack of induction of key costimulatory molecules supports similar findings in the literature and suggests that NIKS cells are incapable of delivering the costimulatory "second signal" essential for activating T cells even in the presence of IFN-γ.[50–52] Furthermore, this result is in agreement with clinical studies of other skin substitutes that indicate a lack of clinical rejection.[53–55] Several cutaneous skin disorders are associated with aberrant expression of HLA-DR on the keratinocyte surface, leading to increased infiltration of inflammatory cells.[56,57] The results presented here indicate that NIKS cells do not express HLA-DR after placement in acute wounds and are therefore unlikely to act as APC leading to acute rejection and enhanced inflammatory responses.

While this study did not investigate the role of minor histocompatibility antigens, the inability of NIKS keratinocytes to induce expression of costimulatory molecules most likely limits their ability to engage in functional T-cell stimulation and induce direct allorecognition. However, indirect allorecognition is an ongoing process due to the continual trafficking of recipient APC through the graft and the turnover of allograft cells. Our data indicate that after placement in full-thickness skin wounds, HLA-ABC was expressed in the majority of stratified keratinocyte layers. We therefore anticipate that like other allogeneic skin substitutes, StrataGraft tissue will not persist permanently on an immunocompetent recipient but will be slowly replaced as the patient's own keratinocytes are able to proliferate and fill in. This is in agreement with previous studies of the Apligraf skin substitute, which showed that it does not permanently engraft and is undetectable after 2 to 3 months.[58–61]

Results from in vitro cellular assays indicated that the patients enrolled in this trial did not develop enhanced proliferative responses to NIKS cells or to allogeneic PBMC after exposure to StrataGraft skin substitute. These data support the conclusion that exposure to StrataGraft tissue does not prime the patient PBMC in vivo. Likewise, no evidence of NIKS-directed cytotoxicity after exposure to StrataGraft tissue was found. It is well established that susceptibility to NK cell lysis is inversely proportional to MHC class I molecule expression on target cells[62–65]; thus, the presence of HLA-ABC molecules on NIKS keratinocytes likely provides protection from cellular lysis by NK cells.[66,67] It is likely that the constitutive presence of MHC class I molecules and the lack of MHC class II and costimulatory molecules reduce the recognition of the allogeneic NIKS keratinocytes as foreign by the patient immune system. In addition, no correlation was observed between increased StrataGraft skin substitute dose and elevated PRA values, nor did patients develop antibody responses to cells of the StrataGraft skin substitute. These in vitro functional assays are indicative of a lack of recipient sensitization to or acute rejection of StrataGraft skin substitute tissue.

Composite human skin substitutes such as those created using the organotypic culturing method outlined here have been suggested to be immunologically neutral, thus providing promise for their acceptability in a grafting environment.[50,55,68,69] While LC are considered the primary APC within cutaneous tissues, it has been established that endothelial cells are also capable of presenting antigen.[70] The tissues described here are devoid of these cell types. Furthermore, we have shown that NIKS keratinocytes do not upregulate costimulatory molecules B7-1 and B7-2, thus limiting the possibility of successful T cell activation even in the presence of inflammatory signals such as those found in the wound environment. Beyond IFN-γ, other immunoregulatory cytokines including IL-1α, IL-6, and IL-12 are insufficient to elicit an alloresponse against NHEK or dermal fibroblasts in functional in vitro assays, further suggestive that keratinocytes can generate an inert skin substitute.[47] StrataGraft skin substitute has unique qualities that make it an invaluable resource for use in epidermal development, tissue engineering, and transplantation studies. The work presented here shows that StrataGraft tissue generated from NIKS keratinocytes is well tolerated and does not elicit an acute immune response in human graft recipients. These findings further substantiate the utility of this unique cell-based product for therapeutic applications in the treatment of cutaneous wounds and disease.

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