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ORIGINAL ARTICLE

Noninvasive evaluation of intragraft immune responses in upper extremity transplantation

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ABSTRACT

In vascularized composite allotransplantation (VCA), invasive tissue biopsies remain the gold standard in diagnosing rejection carrying significant morbidity. We aimed to show feasibility of tape-stripping for noninvasive immune monitoring in VCA. Tape-stripping was performed on allografts and native skin of upper extremity transplant recipients. Healthy nontransplanted individuals served as controls. The technique was also used in swine on naïve skin in nontransplanted animals, native skin of treated, transplanted swine, nonrejecting VCAs, and rejecting VCAs. Extracted protein was analyzed for differences in cytokine expression using Luminex technology. Significantly decreased levels of INFγ and IL-1Ra were seen between human allograft samples and native skin. In swine, rejecting grafts had increased IL-1Ra compared to naïve and native skin, decreased levels of GM-CSF compared to native skin, and decreased IL-10 compared to nonrejecting grafts. Unsupervised hierarchical clustering revealed rejecting grafts separated from the nonrejecting ($P = 0.021$). Variable importance in projection scores identified GM-CSF, IL-1Ra, and IL-2 as the most important profiles for group discrimination. Differences in cytokine expression are detectable in human VCA patient native skin and VCA graft skin using a noninvasive tape-stripping method. Swine studies suggest that differences in cytokines between rejecting and nonrejecting grafts are discernable.

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Key words
cytokine profiling, immune monitoring, noninvasive, skin, rejection, vascularized composite allotransplantation

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Introduction

Vascularized composite allotransplantation (VCA) is a field established over the last two decades that allows restoration of severe tissue defects not amenable for conventional reconstruction [1]. To date, more than 130 hand and upper extremity transplants have been performed with excellent functional and esthetic

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outcomes [2-4]. A unique feature of vascularized composite allografts is their skin component, which is in direct contact with and thus influenced by the outside world. This external component allows for clinical monitoring and often early detection of T-cell mediated rejection (TCMR) episodes, typically presenting as a maculopapular rash on the dorsal and volar aspects of hand and forearm grafts [5,6]. Though the skin component is advantageous for monitoring purposes, it has also been shown that is it's the most immunogenic part of a VCA [7]. This is attributed to the high content of immune-competent cells [8-10] that are crucial to maintain the skin's integrity and to facilitate quick responses to external irritants disrupting its barrier function [11,12]. Though skin alterations can indicate potential ongoing rejection, many other inflammatory or infectious skin conditions can exhibit similar patterns, complicating their differentiation [6,9,13]

The current gold standard for detection of VCA rejection is a punch biopsy of the graft skin and subsequent histopathological grading according to the Banff Criteria [14-17]. However, performing a biopsy is an invasive procedure that can potentially aggravate the immune response [18,19]

TCMR, however, has not only been characterized histologically. As described by Wolfram et al. through investigation of RNA and protein expression in rejecting rat skin, rejection and alloimmune activation leads to upregulation of skin pro-inflammatory cytokines and chemokines like IL-1α, IL-1β, and TNF-α [20]. These expression profiles have the potential to give important information as to the immunologic state of a graft and could, upon refinement, be used as a noninvasive tool to monitor allografts in VCA predicting rejection even before macroscopic alterations emerge.

Tape-stripping is a noninvasive technique that allows collection of protein or RNA from the stratum corneum of the skin by stripping of superficial cell layers [21] It has been shown to be able to extract relevant amounts of protein and RNA for detection of cytokines and chemokines in inflammatory skin conditions such as atopic dermatitis and allergic contact dermatitis [21,22]. Due to its noninvasive nature, this approach could provide a novel immune monitoring method in VCA. This study investigates the use of tape stripping as a tool for immune surveillance in the clinical setting of hand and upper extremity transplantation as well as in a large animal model of VCA.

Materials and methods

All human studies were performed under the approval of the Johns Hopkins Hospital Institutional Review Board (IRB00178542, IRB00194878). All animal studies were performed under the approval of the Johns Hopkins University Animal Care and Use Committee (IACUC).

Experimental groups

Tape stripping in humans was performed on the upper extremity of either healthy control subjects (N_H; $n = 12$) or allograft (G_H) and native skin (T_H) of patients after uni or bilateral hand/forearm/arm transplantation ($n = 4$) (Table S1). None of the transplant recipients included in this study had clinical evidence of rejection at the time of tape stripping. The patient cohort consisted of male transplant patients between the ages 30–55 that had received either unilateral or bilateral upper extremity allotransplantation. All patients had donor bone-marrow infusion post-transplantation and have been on calcineurin inhibitor (tacrolimus) monotherapy since the immediate perioperative period. Tape stripping was further performed on the back of naïve Massachusetts General Hospital (MGH) miniature swine (N_S; $n = 8$), on the native skin (back) (T_S; $n = 6$) or the skin component of tacrolimus-treated nonrejecting heterotopic hind limb allografts (G_S; $n = 5$) in swine that had received an allograft, and on the skin component of untreated rejecting heterotopic hind limb allografts (R_S; $n = 9$) of MGH miniature swine (Table S2). Clinical rejection was scored based on a previously published scoring system [23] Swine hind limb transplantations were performed according to a prior published model [24]

Protein extraction buffer

For extraction of proteins from the D-Squame sampling disks, a protein extraction buffer containing phosphate buffered saline (1%; Sigma-Aldrich, St. Louis, MO, USA), 5% polypropylene glycol (LOT# 0731C130, Amresco®, Solon, OH, USA), 0.1% sodium dodecyl sulfate (SDS 20% solution, LOT# 1711C070, Amresco®, Solon, OH, USA), and one proteinase inhibitor cocktail tablet (Cat. No. 11 836 153 001 Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany) was freshly made and stored at 4°C until use.

D-Squame sampling

Of 5 mL of the protein extraction buffer were put in a 50 mL falcon tube and placed on ice. Eighteen D-Squame

Standard Sampling Discs (#D100; CuDerm Corporation, Dallas, TX, USA) were subsequently applied on the same spot using the D-Squame Angular Tweezers (#D510; CuDerm Corporation, Dallas, TX, USA). After application, pressure was applied for two seconds with the D-Squame® Disc Applicator (#D500; CuDerm Corporation, Dallas, TX, USA). The first D-Squame sampling disk was discarded; the other seventeen were completely immersed in the protein extraction buffer and stored on ice until further processing (Fig. 1).

Protein extraction and concentration

Falcon tubes with immersed D-Squame disks were first placed in a Branson 2800 Series Ultrasonic Cleaner (#35501; Branson Ultrasonics, Danbury, CT, USA) at 4°C for thirty minutes and then centrifuged at 4000xG at 4°C for five minutes. The supernatant was collected and transferred in Amicon® Ultra - 15 ml Centrifugal Filters (Merck Millipore Ltd., Ireland). These were spun for approximately ten to twelve minutes at 4000xG at 4°C. Spinning times varied according to start concentration. This step aimed to reach a concentration volume of approximately 500 µL. The concentrate was collected and transferred to Amicon® Ultra - 0.5 ml Centrifugal Filters (Merck Millipore Ltd., Ireland) to perform a second concentration step. Samples were centrifuged at 14 000xG for four minutes at 4°C. The filter tube was ten turned upside down and spun for additional 20 s to recover the concentrated solution. The concentrate was diluted with 100 µl protein extraction buffer (swine samples were otherwise too concentrated) and transferred in a stock tube and a tube for protein quantification.

Protein quantification

Protein quantification was performed using the Pierce™ BCA Protein Assay Kit (#23227, Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instruction and protein concentrations were measured using a SpectraMax M5e Microplate Reader (Molecular Devices LLC, San Jose, CA, USA).

Luminex analysis

The Bioplex 200 platform (Biorad, Hercules CA) was used to determine the concentration of multiple target

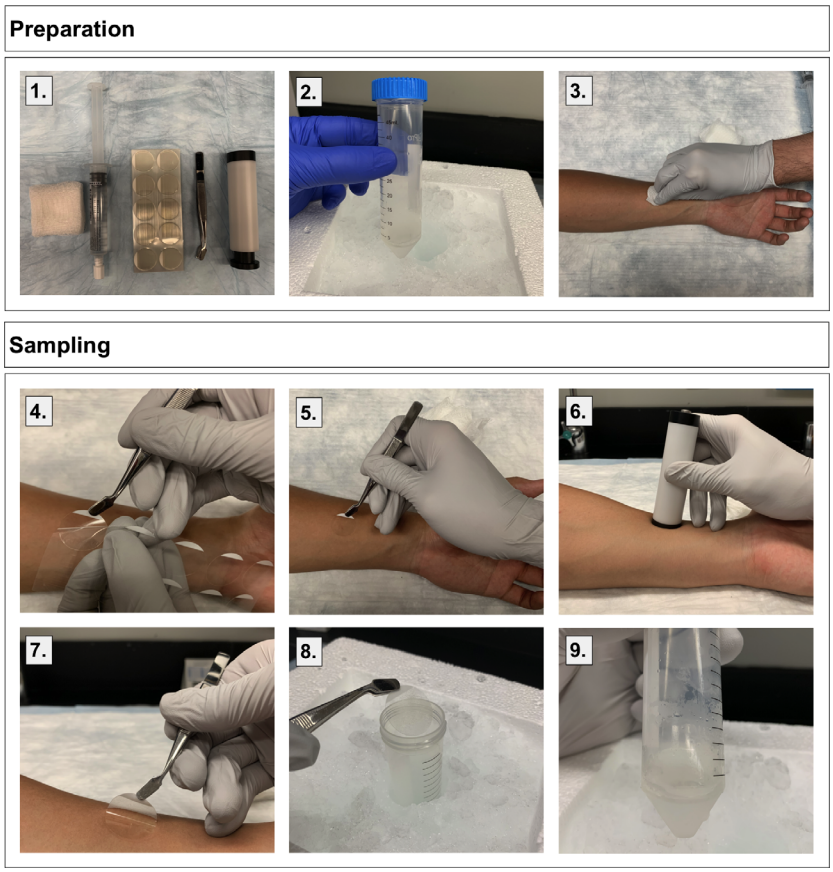

Figure 1 D-Squame Sampling. To collect samples, (1) nonalcoholic solution and gauze along with D-Squame Standard Sampling Discs, Angular Tweezers and Disc Applicator were assembled. (2) Five mL of the protein extraction buffer were put in a 50 ml falcon tube and placed on ice. (3) Skin was gently cleansed with dampened gauze. (4) Eighteen D-Squame Sampling Discs were subsequently applied on the same spot (5) using the D-Squame Angular Tweezers. After application (6) pressure was applied for two seconds. (7) The D-Squame Angular Tweezers were then used to lift the disk off of the skin using the nonadhesive white handling area. The first D-Squame sampling disk was discarded; the other seventeen were completely immersed in the protein extraction buffer (8-9) and stored on ice until further processing.

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proteins in the extracted specimens. Luminex bead-based immunoassays (Millipore, Billerica NY) were performed following core SOPs and concentrations were determined using 5 parameter log curve fits (using Bioplex Manager 6.0) with vendor-provided standards and quality controls. The Invitrogen 10-Plex Porcine ProcartaPlex panel (Thermo-Fisher, Waltham MA) was used to detect GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1Ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α in swine specimens. The HCYTOMAG-60K panel (Millipore) was used to detect IFN-γ, IL-10, IL-12(p70), IL-17A/CTLA4, IL-1Ra, IL-1β, IL-2, IL-4, IL-6, IL-8/CXCL8 in human tape extract specimens.

Histology

Biopsy specimen of rejecting swine (R_S) were fixed in 10% neutral buffered formalin and dehydrated in graded ethanol. Fixed tissues were then embedded in paraffin, sectioned at 5μm, and stained with hematoxylin and eosin (H&E). All slides were reviewed and scored by an expert veterinary pathologist in a blinded fashion based on a previously published scoring system [23]

Statistical analysis

Prism GraphPad 7.0 (GraphPad Inc., San Diego, CA, USA) and the statistical software environment R 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria) were used for statistical analysis. Results are expressed as median and interquartile range (IQR). Mann–Whitney U test or Kruskal–Wallis test with Dunn's correction for multiple comparisons were used. A two-sided *P*-value of less than 0.05 was considered statistically significant. In order to compare levels of individual inflammatory mediators as well as to assess similarity of profiles among different skin types, unsupervised average linkage hierarchical clustering was performed and visualized as heat map using Genesis (version 1.8.1) based on cytokine-wise z-scores (with measurements below detection limit previously set to 1 pg/ml for human assays and 0 pg/ml for swine assays). This was done using Euclidean distance, as commonly used for large scale expression profiling. To test differences of sample proportions between clusters, two-sided Fisher exact test was used. Unsupervised principal component analysis (PCA) was performed on standardized data to underscore group separation. In order to identify the most important contributors to group discrimination, the supervised method partial least square discriminant analysis (PLS-DA) was used. We performed data analysis and result visualization using the R package *mixOmics*, and influence of immune mediator profiles in group discrimination was quantified by the variable importance in projection score (VIP).

Results

Protein extraction

In naïve MGH miniature swine (group N_S), the tape-stripping procedure was able to extract a median of 41 379 μg/ml (IQR 31 965–57 442) protein. Native skin of tacrolimus-treated animals (group T_S) allowed for extraction of 52 024 μg/ml (IQR 38 298–68 071), and tacrolimus-treated nonrejecting graft skin (group G_S) displayed an even higher amount of extracted protein with 65 145 μg/ml (IQR 41 160–75 523). Rejecting (group R_S) allograft skin yielded the highest protein amounts with 73 807 μg/ml (IQR 39 953–99 039) (Fig. 2a). Though a trend toward higher protein concentrations in tacrolimus-treated native and allograft skin as well as in rejecting grafts was seen, differences did not reach statistical significance ($P = 0.4$). Similarly, in animals displaying lower grades of clinical rejection (grade 2, $n = 3$) the amount of protein extracted (median 42 399 μg/ml [IQR 23 951–76 776]) did not show statistically significant difference from that in animals displaying grade 3 rejection ($n = 6$; median 77 094 μg/ml [IQR 47 772–124 832]; $P = 0.2$) (Fig. 2c). Only protein expression of samples with a histological rejection grade > 2B demonstrated an increase protein content compared to tape extract of samples with lower rejection grades (grade ≤ 2B: $n = 4$; median 46 797 μg/mL [IQR 38 729–70 381]; grade > 2B: $n = 4$; median 99 039 μg/mL [IQR 75 450-139.101]; $P = 0.033$) (Fig. 2d).

While naïve skin of control group volunteers (group N_H) allowed for extraction of a median of 10 653 μg/ml (IQR 6244–31 270) of protein and native skin of patients after hand transplantation (group T_H) yielded a median of 18 690 μg/ml (IQR 10 138–39 072), patient graft skin (group G_H) only permitted the retrieval of 5229 μg/ml (IQR 3527–8071) of protein ($P = 0.0006$) (Fig. 2b).

Cytokine and chemokine expression

A detailed overview on absolute cytokine expression is presented in Table 1. In the porcine setting, rejecting

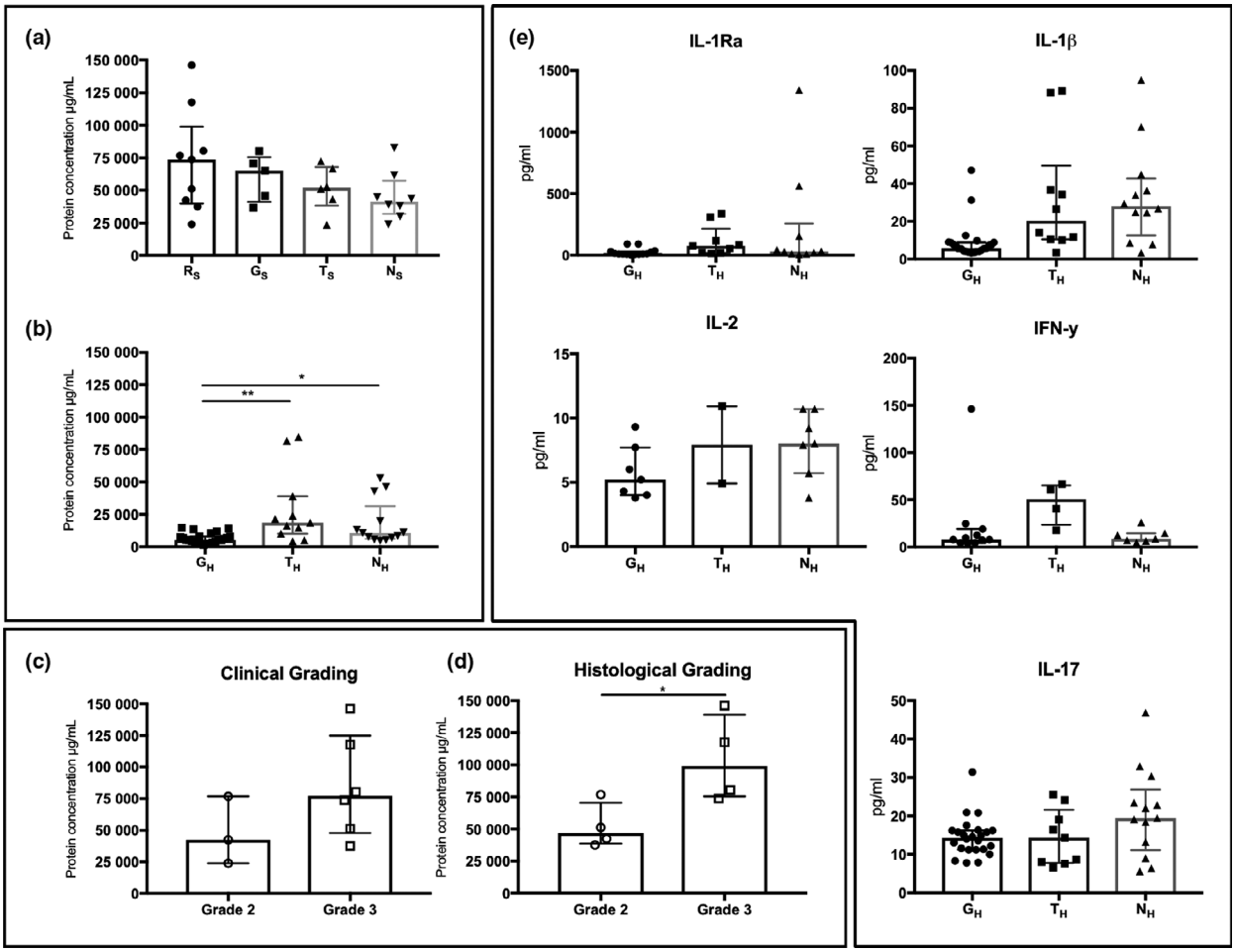

Figure 2 Protein extraction and absolute cytokine and chemokine expression in porcine and human skin analyzed by Luminex technology. Protein extraction in porcine (a, c, d) and human (b) skin using the D-Squame Sampling disks. Median protein levels and interquartile range are shown as measured by the Pierce™ BCA Protein Assay Kit. Absolute cytokine and chemokine expression in human (e) skin was analyzed using the Kruskal–Wallis test and Dunn's correction for multiple comparison. Median levels and interquartile range are shown. $*P < 0.05$; $**P < 0.01$. N, naïve; T, Native skin of tacrolimus-treated subjects; G, nonrejecting tacrolimus-treated graft skin; R, rejecting graft; S, swine; H, human.

grafts (group R_S) displayed significantly higher levels of IL-1Ra compared to groups N_S ($P = 0.0028$) and T_S ($P = 0.0077$). IL-10 expression was higher in group G_S than R_S ($P = 0.027$) and GM-CSF levels were elevated in group T_S compared to group R_S ($P = 0.043$). Similar protein expression levels for IL-1α ($P = 0.099$), IL-1β ($P = 0.955$), IL-2 ($P = 0.069$), IFN-γ ($P = 0.403$), TNF-α ($P = 0.619$), IL-6 ($P = 0.065$), IL-8 ($P = 0.283$), IL-12 ($P = 0.559$), and IL-18 ($P = 0.514$) were measured for all investigated groups. IL-4 expression was below the detection threshold in all samples (Fig. 3). These findings for individual cytokines are evident also from heatmap visualization (Fig. 4a). Unsupervised hierarchical clustering analyses of expression profiles indicated a division of samples into two clusters and revealed that all rejecting grafts (R_S) grouped together into one cluster, separating from the samples of the other cluster with sensitivity of 1.0 and specificity of 0.47 ($P = 0.021$; odds ratio < 0.1; two-sided Fisher exact test) (Fig. 4a). The separation between rejecting grafts and nonrejecting grafts were underscored by principal component analysis (PCA) (Fig. 4b). According to variable importance in projection (VIP) scores from partial least square discriminant analyses (PLS-DA), GM-CSF, IL-1Ra, and IL-2 (VIP > 1) were identified as the most important profiles for the group discrimination (Fig. 4c). Skin samples with a histological rejection grade > 2B demonstrated a significant increase of IL-1b, IL-6, and IL-8 levels compared to samples with rejection grades ≤ 2B (for all three cytokines $P = 0.029$; Figure S1). Similar expression levels were detected for the other investigated cytokines.

Table 1. Comparison of protein extraction (μg/mL; median [IQR]) and cytokine detection (pg/mL; median [IQR]) in different porcine and human study groups

	Marker	N	T	G	R	*P*-value
Swine	Protein	41 379 (31 965–57 442)	52 024 (38 298–68 071)	65 145 (41 160–75 523)	73 807 (39 953–99 039)	0.417
	IL-1Ra	0.23 (0.16–1.29)	0.37 (0.07–1.02)	0.51 (0.27–1.66)	2.07 (1.14–2.49)	0.004
	IL-1α	0.65 (0.27–1.12)	0.49 (0.26–2.36)	1.32 (1.16–2.16)	1.6 (0.98–1.99)	0.099
	IL-1β	0.09 (0.01–0.74)	0.16 (0.09–0.54)	0.01 (0.01–0.01)	0.36 (0.02–0.70)	0.955
	IL-2	0.29 (0.23–0.36)	0.21 (0.15–0.42)	0.46 (0.39–0.50)	0.27 (0.14–0.33)	0.069
	IFN-γ	2392 (1023–5243)	1150 (695.3–4020)	1692 (781.1–3043)	1613 (764.4–1840)	0.403
	TNF-α	22.37 (9.39–35.03)	16.48 (5.04–25.17)	13.64 (8.14–30.25)	10.85 (3.22–24.95)	0.619
	IL-6	0.51 (0.27–0.58)	0.41 (0.28–0.49)	0.19 (0.09–0.20)	0.39 (0.13–0.51)	0.065
	IL-8	1.46 (0.59–4.70)	1.29 (0.51–4.33)	3.03 (1.87–3.95)	2.30 (1.33–10.21)	0.283
	IL-10	0.02 (0.01–0.07)	0.0035 (0.013–0.37)	0.08 (0.05–0.12)	0.02 (0.01–0.03)	0.037
	IL-12	0.05 (0.01–0.06)	0.07 (0.02–0.22)	0.05 (0.04–0.12)	0.04 (0.02–0.06)	0.559
	IL-18	0.21 (0.09–0.44)	0.18 (0.08–0.45)	0.35 (0.23–0.42)	0.18 (0.12–0.19)	0.514
	GM-CSF	0.86 (0.28–1.41)	0.83 (0.62–0.93)	0.63 (0.53–1.01)	0.39 (0.23–0.41)	0.033
Human	Protein	10 653	18 690	5229		0.0006
	IFN-γ	8.70 (6.40–14.50)	50.65 (23.63–65.23)	7.7 (4.20–19.10)		0.040
	IL-17	19.40 (11.10–26.85)	14.30 (7.75–21.55)	14.25 (11.23–16.20)		0.167
	IL-1Ra	31.30 (11.18–257.90)	75.20 (19.14–215.80)	11.20 (8.30–32.10)		0.034
	IL-1β	28.10 (12.63–42.78)	20.35 (10.53–49.55)	5.80 (4.10–8.90)		0.0006
	IL-2	8.00 (5.70–10.70)	7.90 (4.90–10.90)	5.20 (4.00–7.70)		0.307

G, nonrejecting tacrolimus-treated graft skin; N, naïve; T, Native skin of tacrolimus-treated subjects; R, rejecting graft.

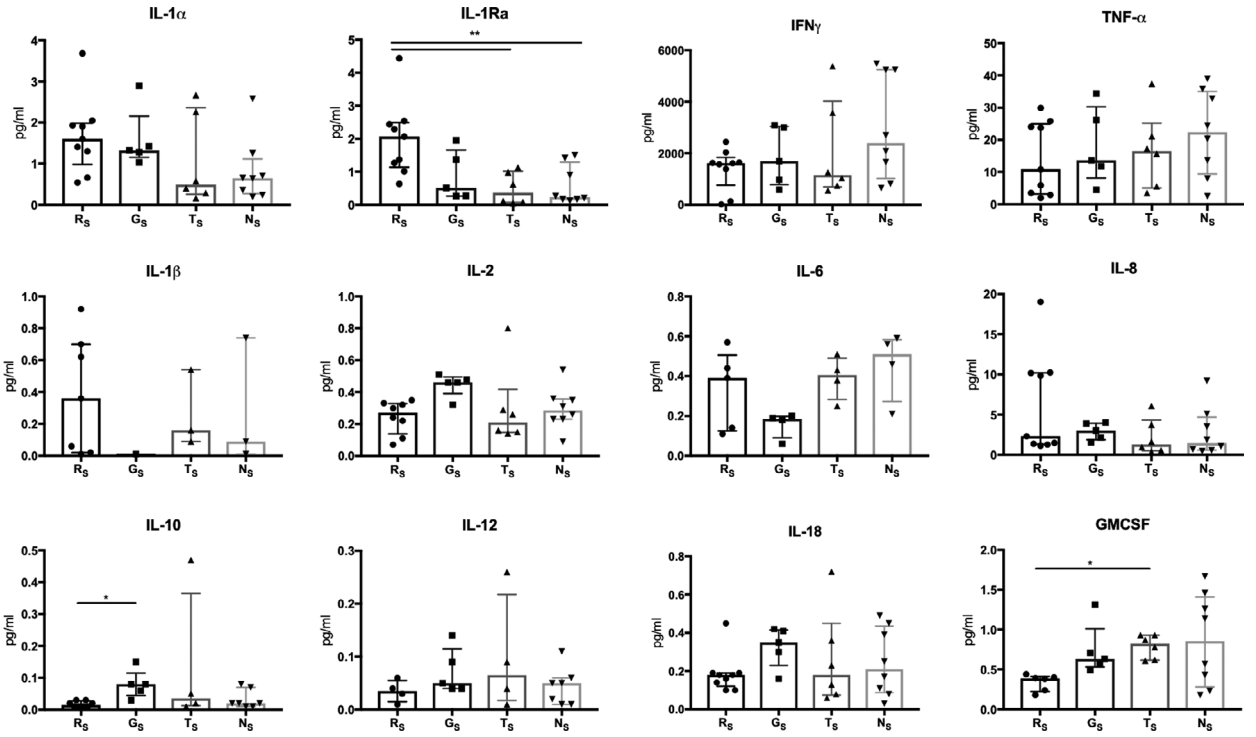

Figure 3 Absolute cytokine and chemokine expression in porcine skin analyzed by Luminex technology. Absolute cytokine and chemokine expression in swine skin was analyzed using the Kruskal–Wallis test and Dunn's correction for multiple comparison. Median levels and interquartile range are shown. *$P < 0.05$; **$P < 0.01$. N, naïve; T, Native skin of tacrolimus-treated subjects; G, nonrejecting tacrolimus-treated graft skin; R, rejecting graft; S, swine.

Samples taken from upper extremity patients were collected as part of routine protocol follow up visits in absence of any clinical signs of allograft rejection. A detailed overview on expression profiles is given in Table 1. Significant differences in expression of IFN-γ ($P = 0.040$) and IL-1Ra ($P = 0.034$) were detectable with highest levels in the T_H group and lowest in the G_H group. IL-1β expression was significantly lower in the G_H groups compared to the other two groups (G_H vs. T_H $P = 0.0081$; G_H vs. N_H $P = 0.0035$). Similar levels of IL-17 ($P = 0.167$) and IL-2 ($P = 0.307$) were detectable (Fig. 2e). Observations of individual cytokines were also confirmed by a heatmap visualization. However, hierarchical clustering analysis did not reveal distinct expression profiles between the different skin groups given the lack of clinical acute rejection episodes (Fig. 5).

Discussion

For patients in whom conventional reconstruction is not feasible, VCA is a validated option to restore form and function after devastating injury and tissue loss [2] However, as with any medical or surgical treatment, the successful procedure carries inherent risks, the most critical of which are side effects from immunosuppression. Though immune response to VCA is different from that of solid organ transplantation [25] the need for immunomodulation still exists. Yet, while side effects of immunosuppression – renal damage, increased cancer risk, infectious complications – are clearly outweighed by the benefits of a life-saving liver transplant, the balance is less objective in the setting of a life-enhancing upper extremity, face, or reproductive organ transplantation. Thus, it is of extreme importance that we are able to prevent rejection using the smallest possible amount of potentially injurious immunosuppression.

Still, with the attempt to decrease exposure to immunosuppressive medications – in particular, calcineurin inhibitors such as tacrolimus – comes the increased risk of rejection. It is the fine balance between preventing rejection and avoiding side effects that is the ultimate goal of those who treat VCA patients. In striving for this perfect balance, there is a need for a better system to monitor for signs of rejection while using minimal immunosuppression [26-29]

Cytokine detection in skin has long been used to evaluate for presence of different skin conditions.

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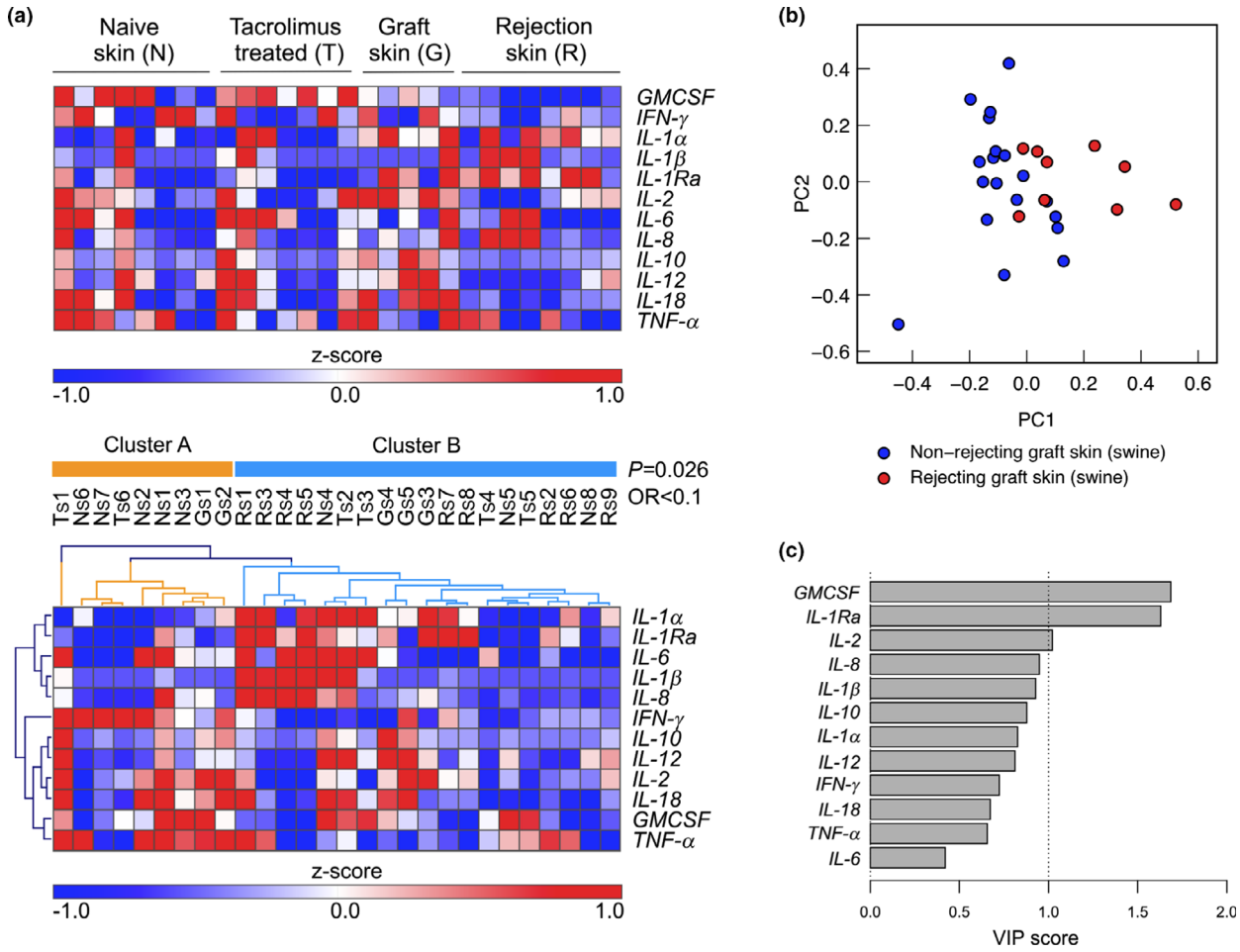

Figure 4 Heatmap, hierarchical clustering and discrimination of rejecting and nonrejecting porcine graft skin. (a) Heatmap and hierarchical clustering of z-scores of porcine tape extracts. Color codes are according to the legend at the bottom, with red indicating high levels of cytokine ($z > 0$) and blue indicating low levels of cytokines ($z < 0$). *P*-value is obtained from two-sided Fisher exact test comparing the proportion of Rs samples in Cluster A versus Cluster B. (b) Principal component analysis (PCA) in a 2D plot with the first two most informative principal components. (c) Variable importance in projection (VIP) scores from partial least squares discriminant analysis (PLS-DA) for discrimination of rejecting and nonrejecting graft skin. Color codes are according to the legend, with red indicating rejecting grafts and blue indicating nonrejecting graft skin. N, naïve; T, Native skin of tacrolimus-treated subjects; G, nonrejecting tacrolimus-treated graft skin; R, rejecting graft; S, swine.

Grellner et al found that IL-1β was detectable at increased levels in early injury, whereas TNF-α was increased after 1–2 h and IL-6 after 24 [30] In inflammatory pathology, cytokines are even more extensively demonstrated to be altered from the control in the setting of different conditions [31-34] These studies give more credence to the concept that identification of dermal inflammatory markers is useful in the care of patients with skin disease.

However, cytokine detection in these studies as well as our current gold standard of immune monitoring through histopathologic analysis utilizes skin punch biopsies. This method contains two main negatives that prevent it from being an ideal monitor: the invasive nature and the potential to incite immune response. Punch biopsies are, without question, procedures, requiring sharp penetration of the graft (multiple times, including local anesthesia injection and suturing). The procedure can be painful, lead to bleeding, and can leave scars. They also require the patient to see the surgeon for monitoring, which, in a procedure that is performed only at specialized centers, can require significant travel and/or effort on the patients' behalf. As well, there have been multiple studies showing that biopsies themselves can actually incite immune responses, thereby causing the specific reaction that they are meant to monitor to help prevent [18,19,35]

Alternatively, tape stripping does not require anesthetic, does not carry the risk of scarring as seen with biopsies, and can be performed repeatedly directly on

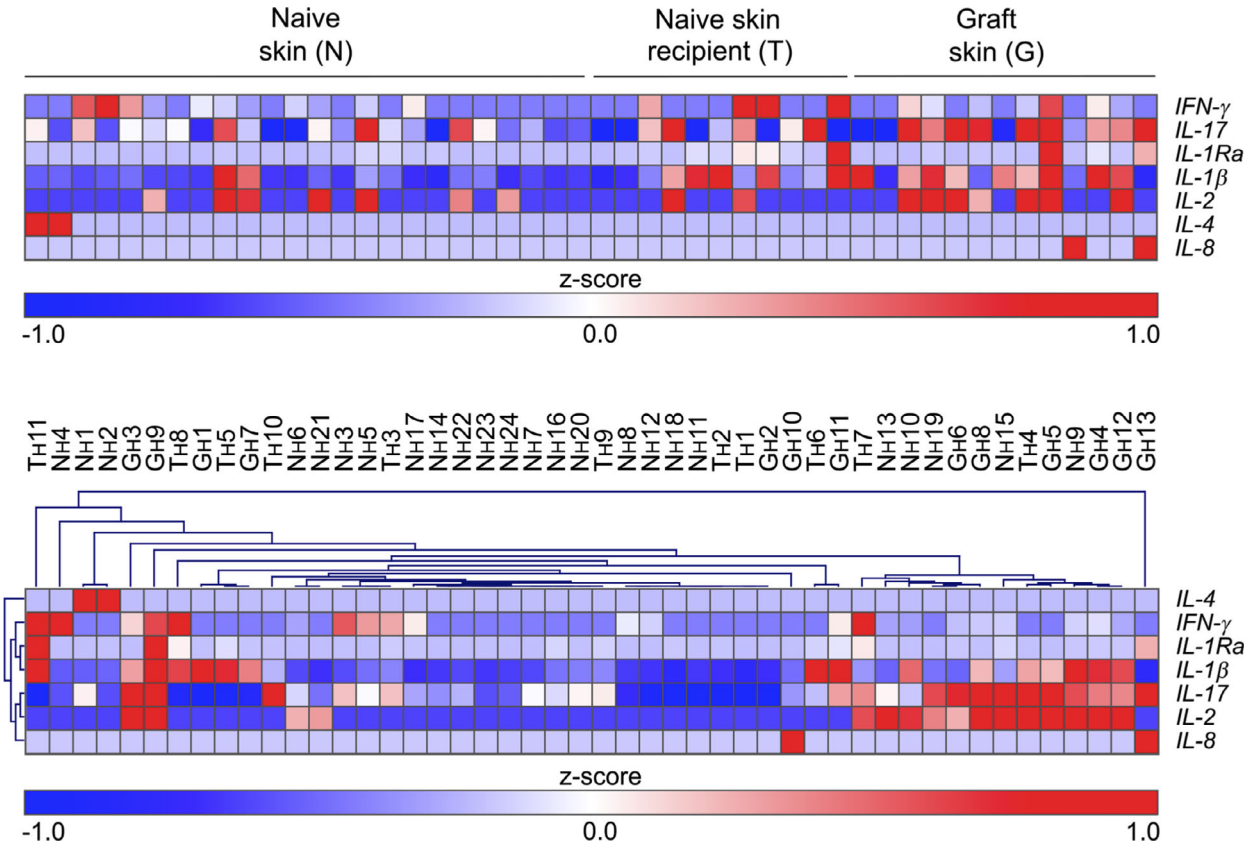

Figure 5 Heatmap and hierarchical clustering of z-scores of human tape extracts. Color codes are according to the legend at the bottom, with red indicating high levels of cytokine ($z > 0$) and blue indicating low levels of cytokines ($z < 0$). N, naïve; T, Native skin of tacrolimus-treated subjects; G, nonrejecting tacrolimus-treated graft skin; H, human.

the lesion or area of interest. As a technique, tape stripping has been well shown to be able to detect protein including specific cytokines through the superficial epidermis [34] Pro-inflammatory cytokines in nontransplant conditions have been shown to be detectable using the method [36] As a small sample, Morhenn et al were able to distinguish irritant pathology from immunologic reaction using tape-stripping detection of cytokine mRNA [22] Perkins et al used tape-stripping and ELISA analysis to detect IL-1α, IL-1Ra, TNFα, and IL-2, and a group out of the United Kingdom has shown that detection of specific cytokines including CXCL1, IL-8, CCL20, and IL36γ was possible by tape stripping, with IL-36γ showing promise for differentiating specific inflammatory immune responses [37]

In VCA, cytokine presence in graft skin has been shown to be associated with graft rejection. Kollar et al utilized an aptamer-based SOMAscan proteomics platform to show alterations in a 5-protein signature during rejection episodes and detected a specific increased in metallopeptidase (MMP3) activity during severe rejection of face transplant [38] The Innsbruck group showed different gene expression of IL-12β, IL-17, and IL-1β in rejection and inflammation of VCA, with CCL7, IL-18, and IL-1β expression being the most indicative of a rejection pathology [39]

Though noninvasive methods have been studied – SOMAscan [38], ultrasound biomicroscopy [40] – to our knowledge, no human-based studies using tape stripping in VCA have been published. A group out of NYU studied in preliminary rodent model the ability to detect different cytokines using a tape-stripping system in rat superficial inferior epigastric flaps, showing an increase of MCP1, MIP3a, and CXCL9 in early rejection and elevated MIP1α, MIP1β, and CXCL10 in advanced rejection with comparable detection of MCP1 and MIP3α in tape-stripping and biopsy samples [41] As well, a study from Tel Aviv mapped inflammatory markers in a rat VCA model using a similar Luminex kit, showing upregulation of IL-18, IFN-γ, CXCL9, 10 and 11, CCL2, CCL5, CX3CL1 and IL-10 in allografts but not in syngeneic grafts [42]

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This study was formulated to show feasibility of an immune monitoring system for VCA that is noninvasive and potentially less triggering for the patient's immune system. Using tape-stripping system that has been previously validated in other skin immunologic processes, we aimed to show the system's capability in detecting systemic and local immune changes seen in graft rejection.

As immunousoppressive treatment itself likely causes modulation in skin expression of cytokines, we opted to compare the skin of untreated control subjects (N_H) with treated nongraft skin (T_H) and graft skin (G_H). This comparison helped us to differentiate expression differences caused by medication from those caused by immune response to a graft. Our results demonstrate that differences in total skin protein levels and specific cytokine expression can be detected in naïve/control skin, native skin of transplant recipients, and allograft skin. In our human subjects, we see that graft skin shows a significantly decreased expression of IFN-γ and IL-1Ra when compared to the expression in the patient's own native skin. This implies that subtle immune differences can, in fact, be detected in human skin using the tape-stripping system. This becomes even more relevant when investigated in the setting of closely monitored immunosuppression and patients without any clinical evidence of rejection. With this study, we have established proof of principle in our human patients, though we did not have the power nor the conditions – including samples of patients with rejecting grafts – required to do a more thorough analysis. After establishing proof of concept, we then used a preclinical large animal study to further investigate this technique.

From the pre-clinical large animal portion of the study, we see that not only can our system detect differences in the baseline expression levels in graft vs. non graft skin, but it can also distinguish cytokine expression patterns from nonrejecting and rejecting grafts. In the swine model, naïve skin (N_S) as well as native skin of treated animals (T_S) show significantly lower level of IL-1Ra expression than that of rejecting grafts. This indicates that the graft, particularly during a stage of acute rejection, expresses levels of IL-1Ra that are far above what is seen in the animal's native skin. In addtion, GMSCF is significantly lower in rejecting grafts than in the native skin, demonstrating further that changes in cytokine expression are specifically due to the graft/rejection state, not the immunosuppressive treatment.

Perhaps more exciting are the differences detected in the swine model between the nonrejecting and rejecting grafts. In our study, we see significantly lower levels of IL-10 in rejecting grafts when compared to those nonrejecting. This decreased expression of IL-10 – an anti-inflammatory cytokine [43-45] – in a rejecting transplant follows the logic that inflammatory upregulation would be present in a graft experiencing a rejection episode. Using the hierarchical clustering analysis, we also demonstrated that the rejecting grafts were found in a separate cluster from the nonrejecting grafts in a statistically significant manner with a sensitivity of 1.0 and a specificity of 0.47. This dichotomy confirms that the measurements obtained were able to successfully distinguish between the level of inflammation seen within grafts. In a subgroup analysis based on histological rejection severity, we could further find significant differences in expression levels of IL-1b, IL-6, and IL-8. The ability to detect these subtle differences in cytokine levels between rejection and nonrejection as well as between rejection severity using the tape-stripping method suggests that – with the correct assay and cytokines – this method has the potential to be applied toward rejection detection in human VCA.

Limitations

This study contains multiple limitations that we would like to acknowledge. First, as this was intended just as a feasibility study, no definite causality can be determined between the changes in cytokine detection. Also, given the constraints in available patients and large animal research, our study lacks power, allowing for the possibility of type II error. Patients were at different timepoints after transplantation (1 to 9 years), which could have contributed to differences in cytokine expression patterns. Along with error, our low number of patients and clinical standard of care dictated that we did not have any human rejection samples. This limited our ability to directly compare expression levels in human grafts based on degree of skin rejection. Cytokine and chemokine detection was more inconsistent in human tape extracts than in swine. While a total of ten proteins were measure, results for more than just single samples were only obtained for five of them, so there could be error in detection that may skew results. Sensitivity and specificity of discrimination of rejection as shown by principal component analysis might not reflect what can be expected in a clinical scenario as it was calculated on a cohort of rejecting and nonrejecting animals. Lastly, given that treatment of the animals prevents graft rejection, our rejecting samples are *without* tacrolimus treatment, preventing a perfect correlation between nonrejecting and rejecting grafts. In addition, differences

in types of rejection and their primary manifestation have to be considered. While tape stripping may be of diagnostic value in the setting of TCMR, antibody mediated rejection, which is also commonly seen in VCA and that does not primarily affect the epidermal layer, might not be detected with this method and thus still requires invasive biopsies for diagnosis.

Authorship

Conceptualization: FM, JWE, GB, BO, GJF, JK, CT. Data Curation: FM, JWE. Formal Analysis: FM, HH, MIL, SB. Investigation: FM, JWE, CT, YG, KK, KA, SAJF, MIL, RK. Methodology: GJF, JK, GB, CT. Supervision: GB, BO. Visualization: FM, HH, RK. Writing - Original Draft Preparation: JE, FM, HH, GB, BC.

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Conflict of interest

The authors have no conflicts of interest to disclose.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Absolute cytokine and chemokine expression analyzed by Luminex technology in porcine skin stratified by grade of rejection

Table S1. Overview human samples.

Table S2. Overview swine samples.

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