WOUND HEALING

A bioengineered living cell construct activates an acute wound healing response in venous leg ulcers

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Chronic nonhealing venous leg ulcers (VLUs) are widespread and debilitating, with high morbidity and associated costs; about $15 billion is spent annually on the care of VLUs in the United States. Despite this, there is a paucity of treatments for VLUs because of the lack of pathophysiologic insight into ulcer development as well as the lack of knowledge regarding biologic actions of existing VLU-targeted therapies. The bioengineered bilayered living cellular construct (BLCC) skin substitute is a U.S. Food and Drug Administration–approved biologic treatment for healing VLUs. To elucidate the mechanisms through which the BLCC promotes healing of chronic VLUs, we conducted a clinical trial (NCT01327937) in which patients with nonhealing VLUs were treated with either standard of care (compression therapy) or the BLCC together with standard of care. Tissue was collected from the VLU edge before and 1 week after treatment, and the samples underwent comprehensive microarray mRNA and protein analyses. Ulcers treated with the BLCC skin substitute displayed three distinct transcriptomic patterns, suggesting that BLCC induced a shift from a nonhealing to a healing tissue response, involving modulation of inflammatory and growth factor signaling, keratinocyte activation, and attenuation of Wnt/β-catenin signaling. In these ways, BLCC application orchestrated a shift from the chronic nonhealing ulcer microenvironment to a distinctive healing milieu resembling that of an acute, healing wound. Our findings provide in vivo evidence in VLU patients of pathways that can be targeted in the design of new therapies to promote healing of chronic VLUs.

INTRODUCTION

Chronic nonhealing venous leg ulcers (VLUs) continue to be a cause of substantial morbidity, straining health care budgets and negatively affecting quality of life. More than 70% of VLUs fail to heal with standard of care compression therapy and have high recurrence rates, posing additional burden to wound care professionals. The chronicity, frequent relapses, and associated complications of nonhealing VLUs heavily affect patients' quality of life and increase health care expenditures for millions of people worldwide.

Deciphering the network of deregulated wound healing processes present in chronic VLUs is challenging, and many therapies showing promise in the laboratory and in initial clinical trials have failed to improve clinical outcomes. The histologic hallmark of chronic VLUs is a hyperproliferative wound edge, which is characterized by nonmigratory keratinocytes, decreased angiogenesis, an increase in proteases, increased bacterial colonization and/or infection, and inflammatory infiltrates (*1*, *2*). We have shown that the nonhealing VLU edge displays loss of genes controlling the fate of local stem cells and their niche, as well as aberrant activation of β-catenin and c-myc (*2*, *3*). Moreover, genomic profiling of VLUs has revealed deregulation of epidermal activation and differentiation, including attenuation of epidermal growth factor (EGF) and transforming growth factor–β (TGF-β) receptor signaling (*4*). However, the molecular pathophysiology of VLUs has not yet been fully elucidated, which has slowed the development and validation of targeted therapies (*5*). There is an urgent need for therapeutic approaches that would target multiple aberrantly regulated cellular processes simultaneously, successfully converting the nonhealing VLU to a healing wound phenotype. Furthermore, enhanced understanding of the molecular pathophysiology of chronic VLUs is critical in identifying relevant clinical trial end points that can be used to evaluate new treatments, paving the way for delivery of maximally efficacious therapies to VLU patients.

A U.S. Food and Drug Administration (FDA)–approved bioengineered bilayered living cellular construct (BLCC) has demonstrated efficacy in promoting healing of chronic ulcers (*6*, *7*). The BLCC skin substitute consists of human foreskin–derived neonatal fibroblasts in a bovine type I collagen matrix below a layer of human foreskin–derived neonatal epidermal keratinocytes. The BLCC has been suggested to interact with the surrounding environment to promote wound healing. In vitro, the BLCC produces growth factors and cytokines that are indispensable for a successful wound healing process (*8*–*10*), but the precise in vivo mechanism of action is unknown. To this end, we designed a randomized controlled postmarketing clinical trial to investigate the effects of a commercially available BLCC (Apligraf, Organogenesis Inc.) on gene expression in chronic VLUs. We analyzed human wound edge biopsies obtained from nonhealing VLUs at baseline and 1 week after BLCC treatment. We hypothesized that treatment with the BLCC might activate responsiveness to cellular signals similar to those that facilitate successful healing of acute wounds, thus changing a nonhealing to a healing phenotype.

RESULTS

Randomized controlled trial of the nonhealing VLU response to BLCC treatment

The study design and participant information for clinical trial no. NCT01327937 is summarized in Fig. 1 and described further in the

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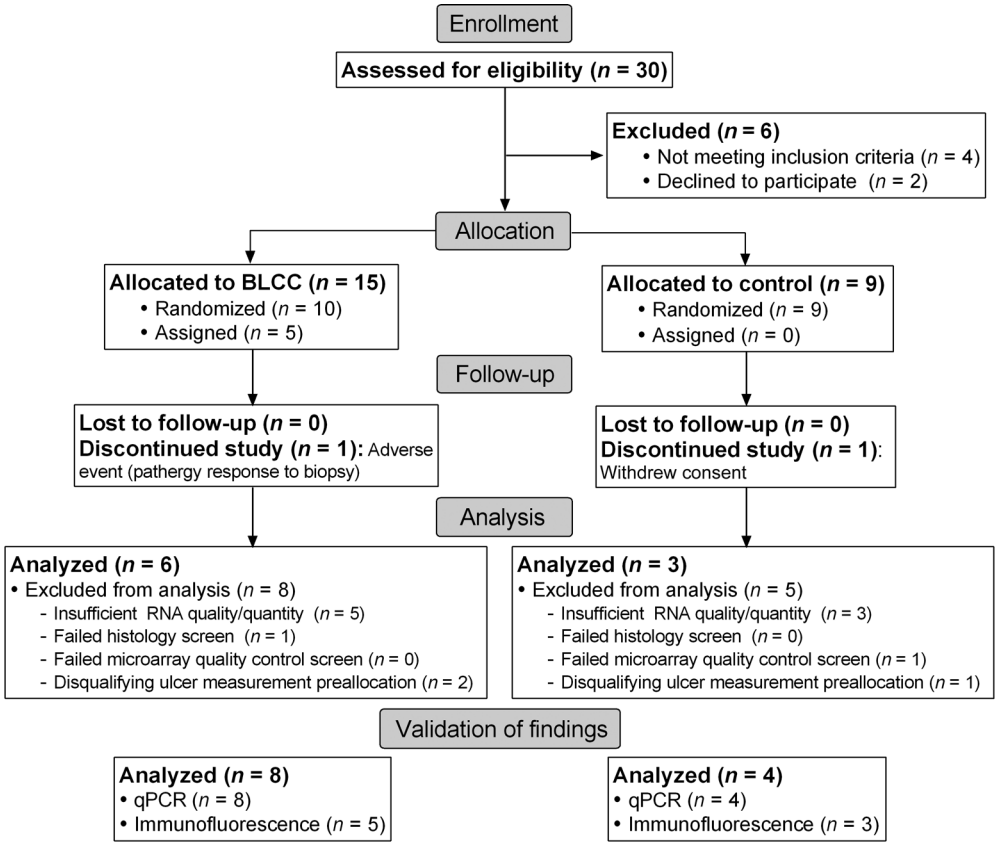


B

	Subject ID	Age (years)	Gender	Race/ethnicity	Ulcer size (cm^2)	
					Screening	Allocation
Control	C1	61	M	White-Hispanic or Latino	17.64	13.26
	C2	41	F	White-Hispanic or Latino	151.7	153.55
	C3	64	F	White-Hispanic or Latino	13.44	16.65
	C4*	83	F	Black or African American	32.43	29.25
BLCC	T1	61	M	Black or African American	46.75	44.0
	T2	55	M	American Indian or Alaska Native	6.8	7.59
	T3	51	F	Black or African American	169.36	161.59
	T4	55	M	White-Hispanic or Latino	26.8	18.36
	T5	45	M	White-Hispanic or Latino	19.74	14.11
	T6	69	M	White-Hispanic or Latino	6.6	12.16
	T7*	84	M	Black or African American	25.53	21.0
	T8*	37	F	Central American–Hispanic or Latino	58.4	42.75

Fig. 1. Participant characteristics. (**A**) Flow diagram of subjects who were assessed and analyzed for the primary outcome of clinical trial no. NCT01327937 (ClinicalTrials.gov). (**B**) Subject demographics. Ulcer size at screening was determined at the time of enrollment (study week −4), whereas size at allocation was determined at week 0, when patients were randomly assigned to BLCC or control treatment groups. Asterisk (*) indicates that the sample biomaterial was of insufficient quality for sensitive microarray analysis but was available for validation of study findings.

Supplementary Materials and Methods. Briefly, potential study participants with VLUs (n = 30) were treated with the standard of care compression therapy for 4 weeks. Patients with nonhealing VLUs, defined as those who did not have a 40% reduction in ulcer size with compression therapy over this time period, were enrolled and randomly assigned to receive either ongoing standard of care treatment with compression dressings (control group, n = 9) or up to five weekly BLCC applications in addition to the standard of care (treatment group, n = 15). Biopsies from the wound edge were obtained at baseline (week 0) and at week 1 for all ulcers, capturing the interval of the first week after study allocation, and ulcer size was monitored over time. At this time point, insufficient RNA quality or quantity was one of the exclusion criteria. Of the patients from whom tissue specimens were obtained, about one-third yielded sufficient high-quality RNA from paired week 0/week 1 biopsies to enable microarray analysis (Fig. 1 and tables S1 and S2).

Immune signals and growth stimuli provided to chronic VLUs by the BLCC skin substitute

The primary outcome measure of our clinical trial was to evaluate changes in the gene expression in wound biopsies from subjects with nonhealing VLUs 1 week after BLCC application compared to the gene expression in wound biopsies from subjects with nonhealing VLUs 1 week after standard of care. To this end, we examined paired microarray profiles of biopsies from the patients with nonhealing VLUs before and after a single application of the BLCC (week 0 to week 1) and compared those to biopsies from the VLU controls receiving standard of care compression therapy (Fig. 1). The peak of the BLCC-mediated effect was expected in the first week of treatment, based on dosing in previous clinical trials (*7*, *8*). To exclude the possibility that BLCC (as opposed to wound tissue) was evaluated, DNA genotypes from week 1 biopsies were examined and compared to cellular genotypes of the BLCC used, to ensure that no cells from the BLCC were detectable in the biopsy specimens at the 1-week time point. We found 559 microarray probes (corresponding to 424 unique genes) significantly differentially expressed between week 0 and week 1 after BLCC treatment, compared to differential expression in control biopsies, which showed a change in only 92 probes (70 genes) (paired t test, $P < 0.05$; fold change, >1.5). There were five genes

that overlapped between BLCC-treated and control biopsies (Fig. 2A and table S3).

Although some variability in gene expression among individual study subjects was evident, a clear BLCC-specific consensus transcriptional signature emerged (fig. S1). Gene ontology analysis of differentially expressed entities after BLCC application highlighted a strong enrichment in immune system biological processes, with 18.1% (101 of 559) of the modulated genes related to the immune response ($P = 2.83 \times 10^{-26}$) (Fig. 2B). The highly enriched biological processes also included "regulation of response to wounding," as well as "epithelium development," consistent with the demonstrated clinical role for the BLCC in accelerating wound healing.

Ingenuity Pathway Analysis (IPA) of the differentially expressed genes after BLCC treatment identified multiple enriched pathways (Fig. 2C and table S4), all of which are related to various aspects of the innate and adaptive immune response. In contrast, in the standard of care VLUs, no significantly enriched pathways were identified using this approach. Using published algorithms (*11*), IPA Core Analysis generated predicted networks connecting upstream regulators to downstream biological processes that were significantly enriched among the BLCC-stimulated genes. One such network is shown in Fig. 2D, in which the upstream regulator interleukin-1β (*IL1*β) induced a set of chemokines, which was significantly up-regulated after BLCC treatment, to increase chemotaxis, an enriched and activated biological process (Fig. 2B). These pathway and network data provide initial support for the hypothesis that BLCC application introduces an alternate inflammatory response distinct from chronic inflammatory infiltrates typically found in nonhealing VLUs (*12*).

IPA analysis also predicted multiple upstream regulators whose targets were enriched among BLCC-modulated genes, including such key wound healing factors as TGFβ, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) (Fig. 2E). Moreover, the directionality of downstream gene expression (that is, induction versus inhibition) supported active signaling states of these growth factors (table S5 and fig. S2).

Generation of acute and chronic wound reference gene expression profiles

We hypothesized that the inflammation triggered by BLCC application was similar to that invoked during the acute wound healing response. To explore this possibility, we needed to differentiate the inflammation during acute wound healing, which leads to successful wound closure, from the chronic inflammation present in the background of nonhealing VLUs. To do so, we generated three reference gene expression profiles comparing intact skin, acute wounds at day 3 after wounding, and chronic VLUs (Fig. 3A and table S7). We used these reference gene expression profiles to explore possible mechanisms through which application of BLCC might uniquely shift chronic nonhealing VLUs to an acute wound healing–like phenotype.

To obtain an acute wound healing profile that could be coordinately analyzed head to head with our chronic VLU profiles, we used the raw in vivo full-thickness acute wound microarray data previously generated from human skin graft donor site wounds [Gene Expression Omnibus (GEO) accession no. GSE28914 (*13*)]. We downloaded paired data from six patients, comparing intact versus healing skin at day 3 after wounding (*13*), to capture the early response and inflammatory phase of acute wound healing (acute versus intact; Fig. 3A and tables S6 and S7). A reference expression profile of the prototypic nonhealing chronic VLUs was obtained from the pretreatment (week 0) biopsies of three patients with VLUs who displayed poorest healing trajectories during the 4-week screening period before randomization (fig. S3; patients C2, C3, and T6), as compared with biopsies of healthy (intact and unwounded) skin (chronic versus intact; Fig. 3A and tables S6 and S7). Finally, we compared the acute wound profiles to transcriptional profiles of the baseline chronic VLUs to enable discrimination of the acute response from the background of chronic VLU inflammation (acute versus chronic; Fig. 3A and table S6). The three reference profiles contained distinct and overlapping genes (Fig. 3A).

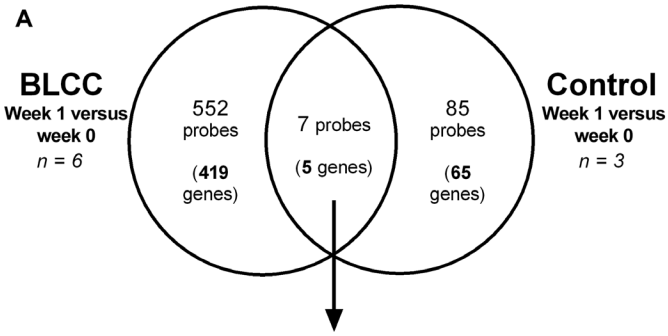
We used these reference profiles to propose the following mechanisms for BLCC effects. For genes modulated in both acute and chronic wounds, changes may occur in expression but in opposite directions when compared to intact skin, but BLCC treatment might revert the expression in VLUs toward the acute wound phenotype (Fig. 3B, "a"). For genes with consistently altered expression in both acute wounds and chronic VLUs, but pathologically over- or underexpressed in VLUs, the BLCC may normalize this perturbed gene expression back to expression levels observed in acute healing wounds (Fig. 3B, "b"). For genes modulated during acute wound healing, but not in chronic VLUs, the BLCC may regulate the response in the direction consistent with acute wound levels, which are associated with a beneficial healing outcome (Fig. 3B, "c").

BLCC reversal of gene dysregulation in nonhealing chronic VLUs toward an acute wound healing phenotype

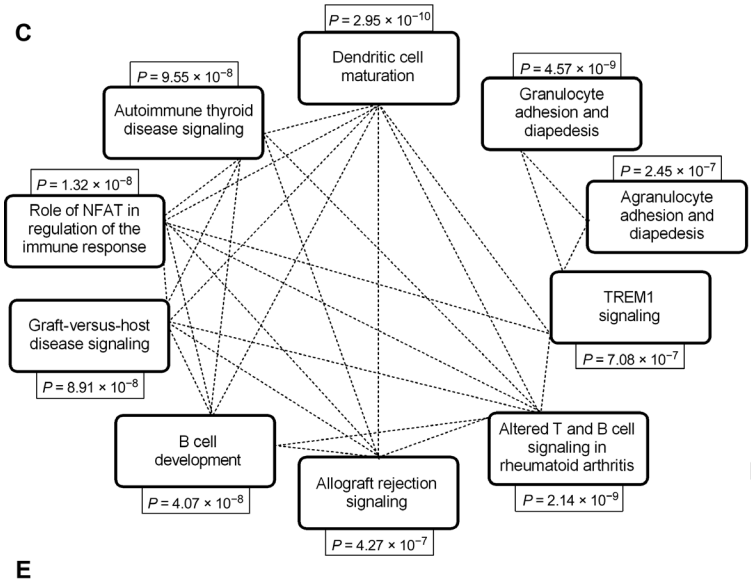
We proceeded to examine the BLCC and control gene expression profiles (week 1 versus week 0; table S3) for evidence of the proposed mechanisms in Fig. 3B. We identified caspase-14 (CASP14) as a BLCC-modulated gene representing model a in Fig. 3B. CASP14 is a nonapoptotic caspase with a critical role in the terminal differentiation of keratinocytes (*14*). As expected, its expression was lower in acutely healing wounds and, consistent with previous reports (*4*), was higher in nonhealing chronic VLUs (Fig. 4A). *CASP14* transcript levels were down-regulated in VLUs after BLCC treatment, adopting the direction of acutely healing wounds (Fig. 4A).

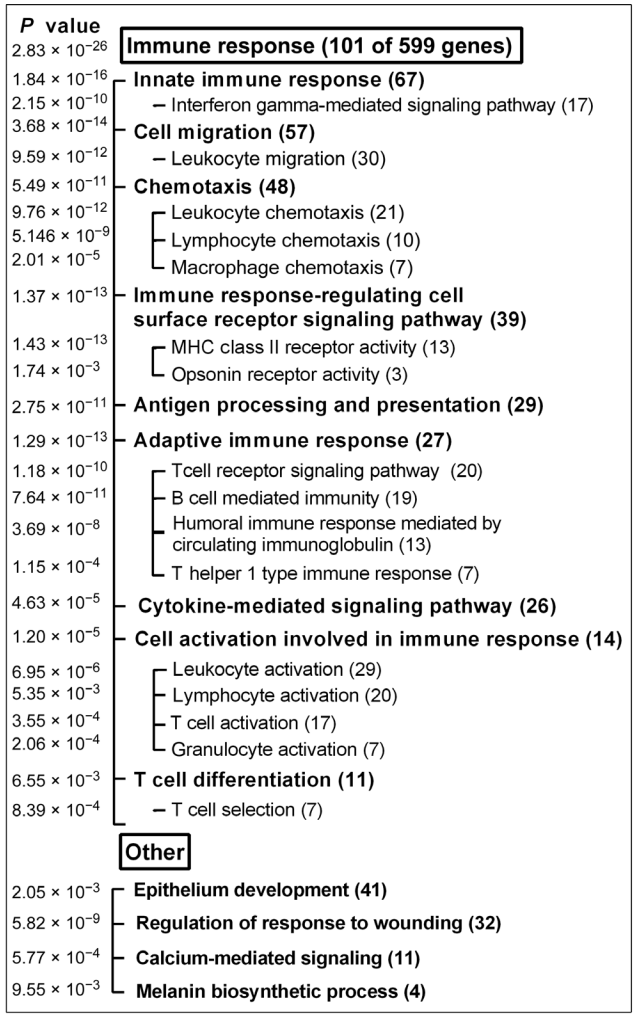
Model b in Fig. 3B was represented by the expression of integral membrane protein 2A (*ITM2A*) and type 2 thyroid deiodinase (*DIO2*) (Fig. 4, B and C). ITM2A is an integral transmembrane protein that is variably expressed in T cell lineage hematopoietic cells, with high expression in select subsets of activated T cells and lower expression in stimulated regulatory T cells (*15*, *16*). BLCC application induced expression of *ITM2A* in the chronic VLUs to an expression level consistent with an acutely healing wound. *DIO2* was induced during acute wounding and even further overexpressed in chronic nonhealing VLUs (Fig. 4C). DIO2 catalyzes the conversion of thyroid hormone to its active form (T_3) and functions as the key regulator of thyroid hormone action on target tissues, including the skin (*17*). Thyroid hormone broadly influences epidermal development and function and has a demonstrated role in wound healing (*18*). *DIO2* expression in BLCC-treated VLUs was similar to that in healing acute wounds (Fig. 4C). *ITM2A* and *DIO2* expressions were unchanged in the VLUs from control patients receiving the standard of care; none of the genes expressed in the group receiving compression therapy alone showed the expression pattern in model b of Fig. 3B (table S3).

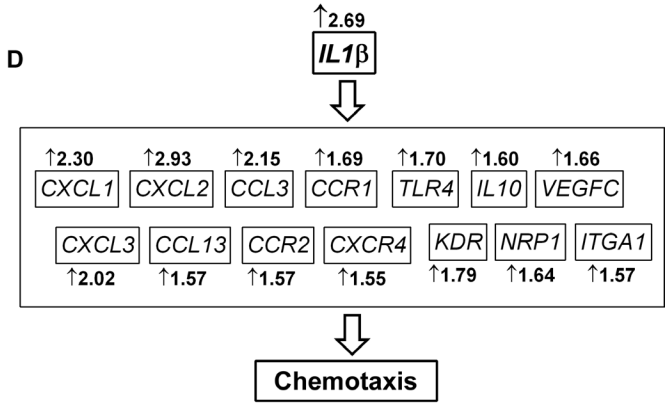
We identified multiple up- and down-regulated genes (Fig. 4D) corresponding to model c of Fig. 3B. Principal components analysis



Affymetrix probe ID	Gene symbol	Fold change week 1 versus week 0	
		Control	BLCC
236203_at	HLA-DQA1	1.55	1.54
207433_at	IL10	1.95	1.60
206391_at	RARRES1	1.79	1.72
206392_s_at	RARRES1	2.38	2.42
221872_at	RARRES1	2.76	2.79
205152_at	SLC6A1	−1.83	1.53
221561_at	SOAT1	1.65	1.59







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Activated growth factor	Number of downstream genes	Overlap *P* value	Activation score
TGFB1	78	1.68×10^{-16}	2.48
HGF	15	2.71×10^{-2}	2.05
VEGFA	12	5.29×10^{-4}	2.53
PDGFA/B	12	1.08×10^{-2}	2.94

Fig. 2. Transcriptional response of chronic VLUs to BLCC treatment or compression therapy. (**A**) Venn diagram of significantly regulated probes and the corresponding genes in BLCC- and control-treated VLUs (fold change, >1.5; paired *t* test, $P < 0.05$). (**B**) Gene ontology analysis of enriched biological processes among the BLCC-modulated genes. Using the Broad Institute Gene Set Enrichment Analysis (GSEA) algorithms, significantly overrepresented functions are grouped by category and listed with enrichment *P* values (left column) as well as the number of corresponding BLCC-influenced genes (parentheses). (**C**) IPA of BLCC-modulated genes. The top 10 pathways are shown, with the corresponding Benjamini-Hochberg–corrected enrichment *P* values; dotted lines represent genes that are common to the linked pathways. See also table S4. (**D**) IPA-predicted mechanistic network for BLCC stimulation of immune cell chemotaxis via *IL1*β; fold changes in *IL1*β target gene expression after BLCC treatment are shown. (**E**) Growth factors with IPA-predicted active downstream signaling in response to BLCC treatment, as indicated by the overlap of their known targets with BLCC-modulated genes (Fisher's exact test, $P < 0.05$) as well as by an activation (*Z*) score of >2, reflecting consistent gene expression changes in response to BLCC treatment (*11*). See also fig. S2 and table S5.

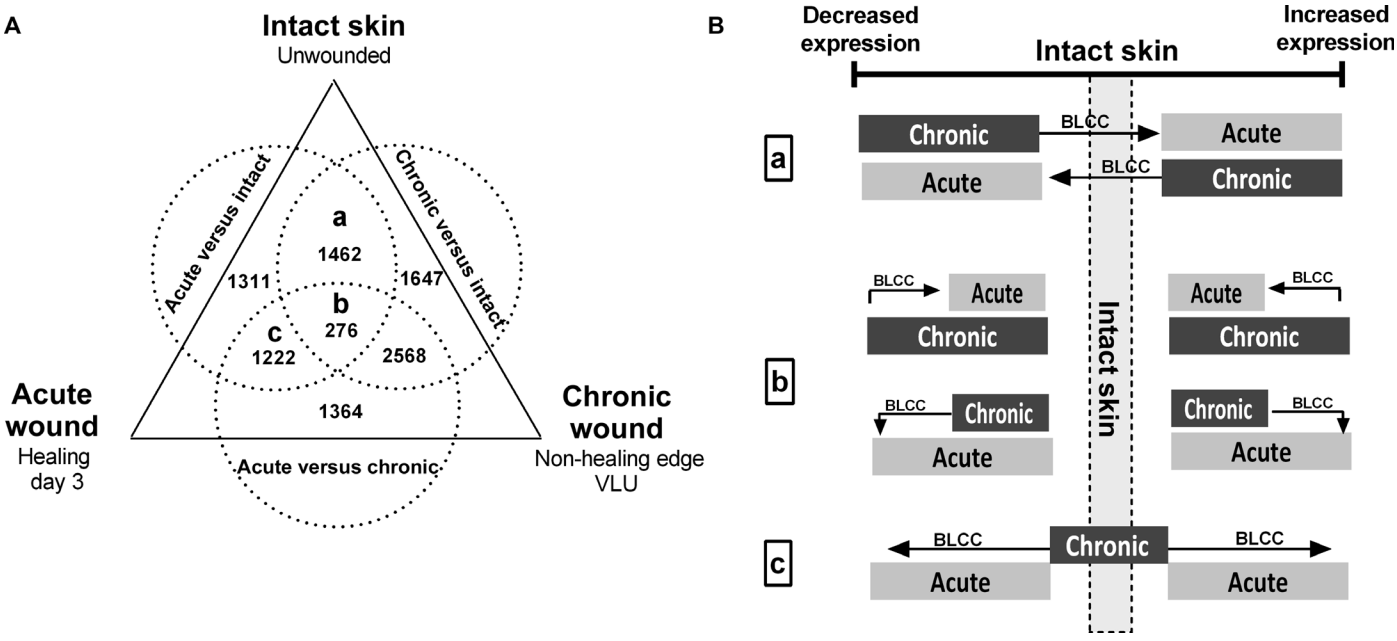

Fig. 3. Models for BLCC-triggered acute wound healing response. (**A**) Venn diagram of three reference profiles generated using microarray data from intact unwounded skin (n = 6), healing acute wounds at day 3 after injury (n = 6), and the nonhealing edge of chronic VLUs (n = 3), as described in tables S6 and S7. Letters a, b, and c indicate aggregates from which genes in Fig. 2B were obtained. (**B**) Proposed mechanisms by which BLCC might accelerate healing of chronic VLUs: (a) reversing expression of genes with divergent expression in acutely healing wounds versus chronic VLUs, (b) shifting genes that are pathologically hyperregulated in chronic VLUs back to acute healing wound levels, or (c) inducing expression of genes modulated in acute healing wounds that are quiescent in chronic VLUs.

(PCA) demonstrated a shift in BLCC-treated VLUs, but not in control VLUs, toward a gene expression profile—consistent with acute wounds during the inflammatory wound healing phase (day 3) (Fig. 4E). Moreover, when plotted against each other, changes in gene expression in acute wounds correlated significantly with changes in gene expression in BLCC-treated VLUs (Fig. 4F).

Recapitulation of biological processes of acute wound healing by BLCC treatment

Pathway analysis also supported the global hypothesis that BLCC treatment, in contrast to standard of care compression therapy alone, reverted the chronic nonhealing VLUs to an acute wound healing phenotype. We performed IPA on the reference gene profiles established in Fig. 3A and compared them with biological pathways enriched in BLCC- and control-treated VLUs (week 1 versus week 0) (see tables S3 and S7). Although a small number of genes were modulated in both BLCC- and control-treated VLUs, acute wound healing biologic pathways including innate and adaptive immunity and their interaction were enriched exclusively in acute and BLCC-treated VLUs (Fig. 5A and table S8). Biological processes of B cell proliferation, antibody production, calcium mobilization, and T cell differentiation, activation, migration, and signaling were present in BLCC-treated wounds but absent in chronic VLUs at baseline or after compression treatment (Fig. 5B and table S9).

BLCC treatment-induced acute inflammatory wound healing in chronic VLUs

To validate our microarray findings, we used Ingenuity Knowledge Base to identify a literature-supported network of genes and biological processes enriched in acute healing and BLCC-treated wounds but not chronic VLUs at baseline or after standard of care treatment (Fig. 6A). An expanded pool of biopsies from BLCC-treated (n = 8) and control-treated (n = 4) VLUs was used to validate the expression of *IL1*β, *CXCL1*, *CXCL2*, and *CXCL3* using quantitative polymerase chain reaction (qPCR) (Fig. 6B). We used a well-characterized ex vivo model of acute wound healing (*19*) using healthy human skin samples (n = 2) to confirm the induction of all four genes during the acute wounding response (Fig. 6C and fig. S4). Qualitative immunofluorescence analysis of protein tyrosine phosphatase receptor type C (PTPRC), which encodes the CD45 receptor expressed on the surfaces of leukocytes, identified an increased dermal infiltrate of $CD45^+$ cells in BLCC-treated tissue samples (Fig. 6D). We confirmed that BLCC treatment induced Toll-like receptor 4 (TLR4) expression in the upper spinous and granular layers of the epidermis (Fig. 6E). TLR4 functions in both innate and adaptive immunity and is an important player in the inflammatory phase of early wound healing (*20*).

Attenuated Wnt/β-catenin signaling in healing BLCC-treated VLUs

The BLCC skin substitute has demonstrated efficacy in healing chronic ulcers in conjunction with standard therapy (*6*, *7*), but VLUs that do not respond to initial applications of BLCC are unlikely to derive additional clinical benefit from repeated BLCC treatments (*21*). To determine whether gene expression could be retrospectively correlated with a successful 4-week healing trajectory, we performed regression analysis on the healing curves of the control- and BLCC-treated VLUs from week 0 through week 4 (Fig. 7). We identified changes in gene expression from week 0 to week 1 in the BLCC-treated VLUs that correlated with the trajectory slope of the "healers" but not with the slope of the "nonhealers" or controls receiving compression therapy alone (table S10). One of these genes was *CSNK2A2*, a casein kinase with an essential role in Wnt/β-catenin signaling (*22*). BLCC

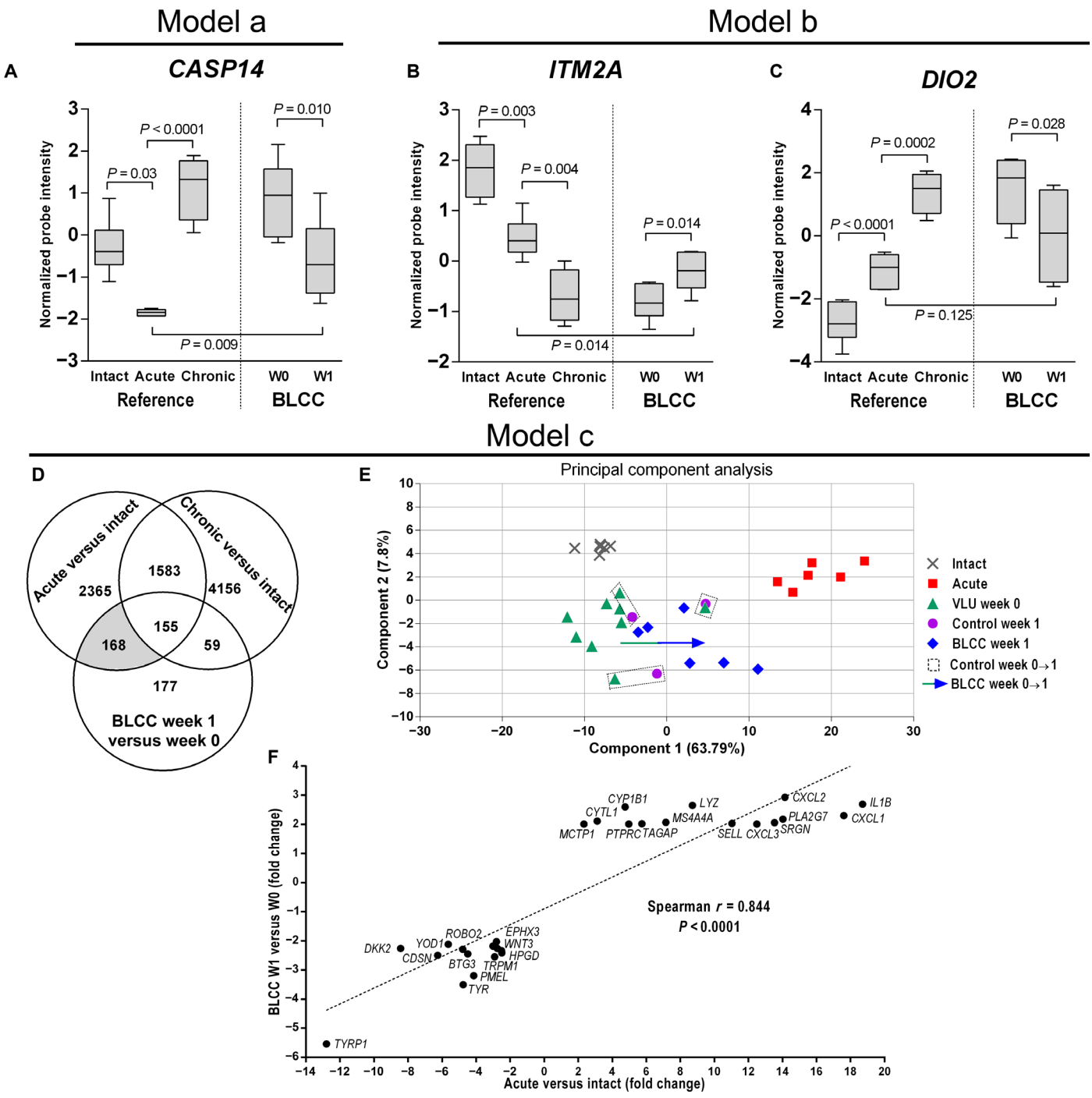

Fig. 4. Model validation for BLCC-induced gene expression changes in chronic VLUs. Expressions of gene aggregates identified in Fig. 2A were explored in the context of the models proposed in Fig. 2B by comparing reference profiles of intact unwounded skin, healing acute wounds at day 3 after injury, and the nonhealing edge of chronic VLUs to pairs of nonhealing VLUs before and after BLCC treatment. Data are represented as box- and- whisker plots of microarray probe expression intensities in the unwounded skin ("Intact," $n = 6$), healing acute wounds ("Acute," $n = 6$), nonhealing chronic VLUs ("Chronic," $n = 3$), as well as six pairs of BLCC-treated VLUs ("W0" and "W1"). (**A**) Model "a": *CASP14* expression in intact, acute, and chronic reference samples compared to BLCC-treated VLUs at W0 and W1. (**B** and **C**) Model "b": *ITM2A* and *DIO2* expression in intact, acute, and chronic reference samples compared to BLCC-treated VLUs at W0 and W1. *P* values were determined by two-tailed paired *t* test (acute versus intact; BLCC W1 versus W0) or two-tailed moderated *t* test (acute versus chronic; BLCC W1 versus acute). (**D** and **E**) Model "c": Four-component PCA of 168 microarray probes common to healing acute wounds and chronic VLUs after BLCC (shaded in gray); plot of components 1 and 2 comprise over 70% of the variation. Arrow represents the trend toward acute wound expression in 1 + PCA components for pairs of BLCC-treated VLUs (BLCC week 0 to 1), which is absent in control-treated pairs (dotted rectangles). (**F**) Plot of changes in the gene expression for BLCC-treated VLUs (fold change; W0 to W1) and changes in the gene expression between acute wounds and intact skin as determined by Spearman's nonparametric rank order correlation (two-tailed $P < 0.0001$).

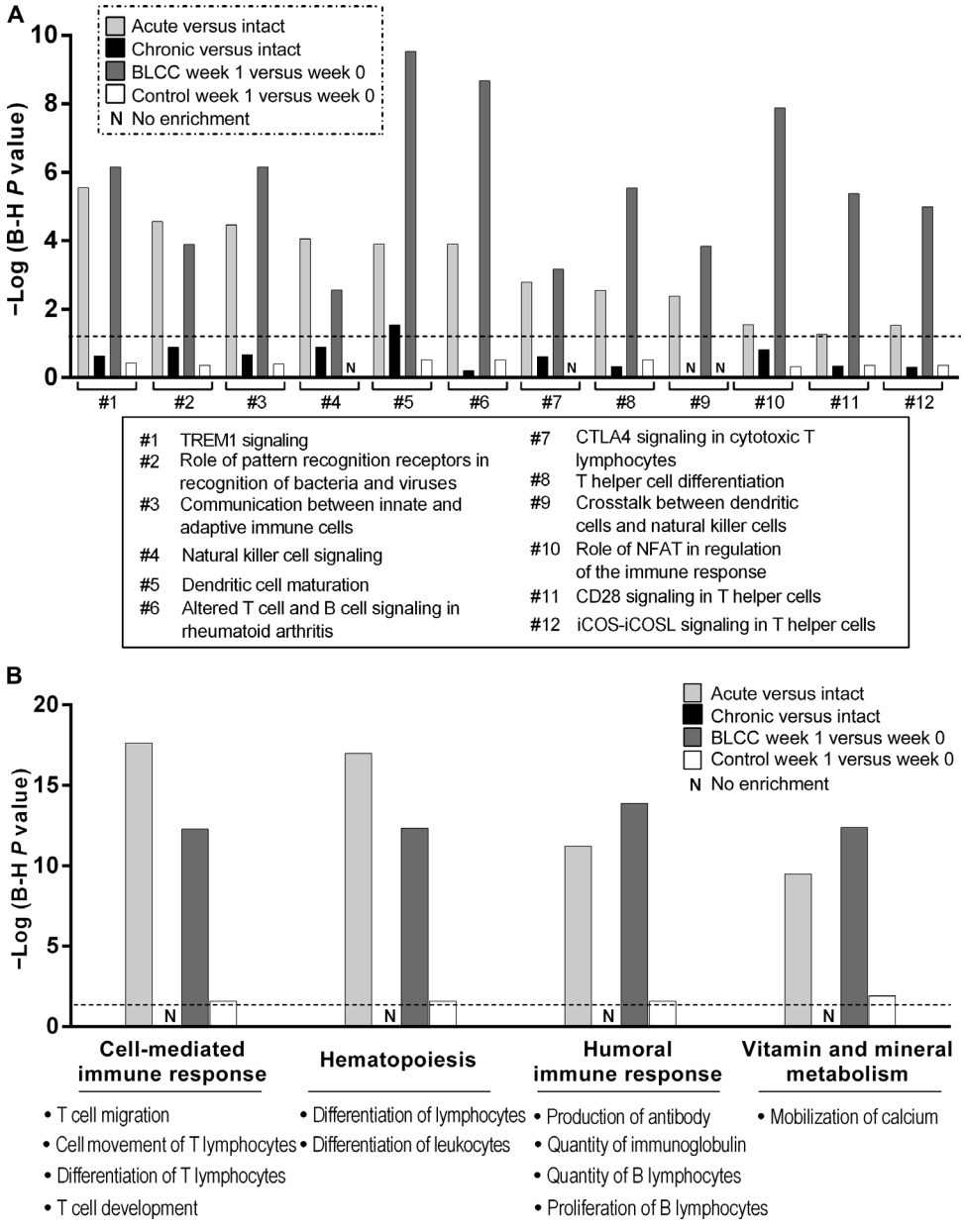

Fig. 5. Biologic pathways and processes common to acutely healing wounds and BLCC-treated VLUs. (**A**) IPA identified pathways and (**B**) biologic processes significantly enriched in acutely healing wounds (acute versus intact, $n = 6$ pairs) and in chronic VLUs after 1 week of BLCC application (BLCC week 1 versus week 0, $n = 6$ pairs) but not enriched in VLUs treated with standard of care compression alone (control week 1 versus week 0, $n = 3$ pairs) nor in nonhealing VLUs at baseline [$n = 3$ chronic VLU versus $n = 8$ intact (unwounded) skin]. Histograms reflect P values of enrichment for each pathway or process assessed by Fisher's exact test after Benjamini-Hochberg (B-H) correction for multiple testing. Dotted line denotes thresholds for significance at Benjamini-Hochberg $P = 0.05$. See tables S8 and S9 for corresponding genes.

treatment decreased *CSNK2A2* expression in VLUs, and the magnitude of down-regulation correlated with the healing extent in the healers [$R^2 = 0.9980$ (correlation); $P = 0.02$] but not in nonhealers and/or control VLUs (Fig. 7A). Reduced expression of *CSNK2A2* is a shared feature of BLCC-healing VLUs and acute wound healing because *CSNK2A2* expression decreased in vivo by about threefold at 72 hours after wounding (Fig. 7B and table S7, acute versus intact). Furthermore, *WNT3*, which regulates CSNK2A2 activity (*22*), decreased in BLCC-treated chronic VLUs and in in vivo and ex vivo models of acute wound healing (Fig. 7C). Finally, although immunofluorescence of phosphorylated β-catenin in nonhealing VLUs confirmed previously described intense nuclear staining in wound edge keratinocytes (*2*), nuclear β-catenin was absent in portions of BLCC-treated healing VLUs (Fig. 7D).

DISCUSSION

Our clinical trial was designed to understand the mechanisms of action of an FDA-approved skin substitute that has already demonstrated clinical efficacy in healing chronic ulcers (*6*). We found that BLCC application induces genes and processes of acute inflammatory healing, activates keratinocytes, and diminishes Wnt/β-catenin signaling, thus recapitulating features of the acute wound phenotype. Our data therefore suggest that therapeutic approaches that can successfully activate an acute wound healing response have the greatest likelihood of being efficacious in the clinical setting. Hence, our findings provide representative testable genomic and molecular end points that can be incorporated into the design of the future clinical trials testing the efficacy of VLU therapies.

Our data demonstrate that BLCC application triggers an inflammatory response that is distinct from the chronic inflammation present in nonhealing VLUs at baseline. It is unlikely that we are capturing acute rejection of the allogeneic cells of the BLCC, that is, graft-versus-host response, for several reasons. First, well-documented clinical signs of rejection, such as pain, erythema, and necrosis, are absent from both acute and chronic wounds that have been treated with BLCC (*6*, *23*). Second, the BLCC does not contain resident skin antigen–producing cells, such as Langerhans cells or melanocytes, and keratinocytes and fibroblasts do not express class II human leukocyte antigens (HLAs) or other immune costimulatory molecules (*6*). Third, antibodies signifying a BLCC-specific immune rejection (for example, anti-HLA type I BLCC alloantigens) were not detectable in VLU patients treated with BLCC (*6*).

Rather than reflecting a rejection response, comparison of the BLCC gene expression profile to in vivo acute wound profiles illustrated that this BLCC-invoked inflammation recapitulated features of the inflammatory phase of acute wound healing, which is an essential stage of successful wound closure. We demonstrated this at the

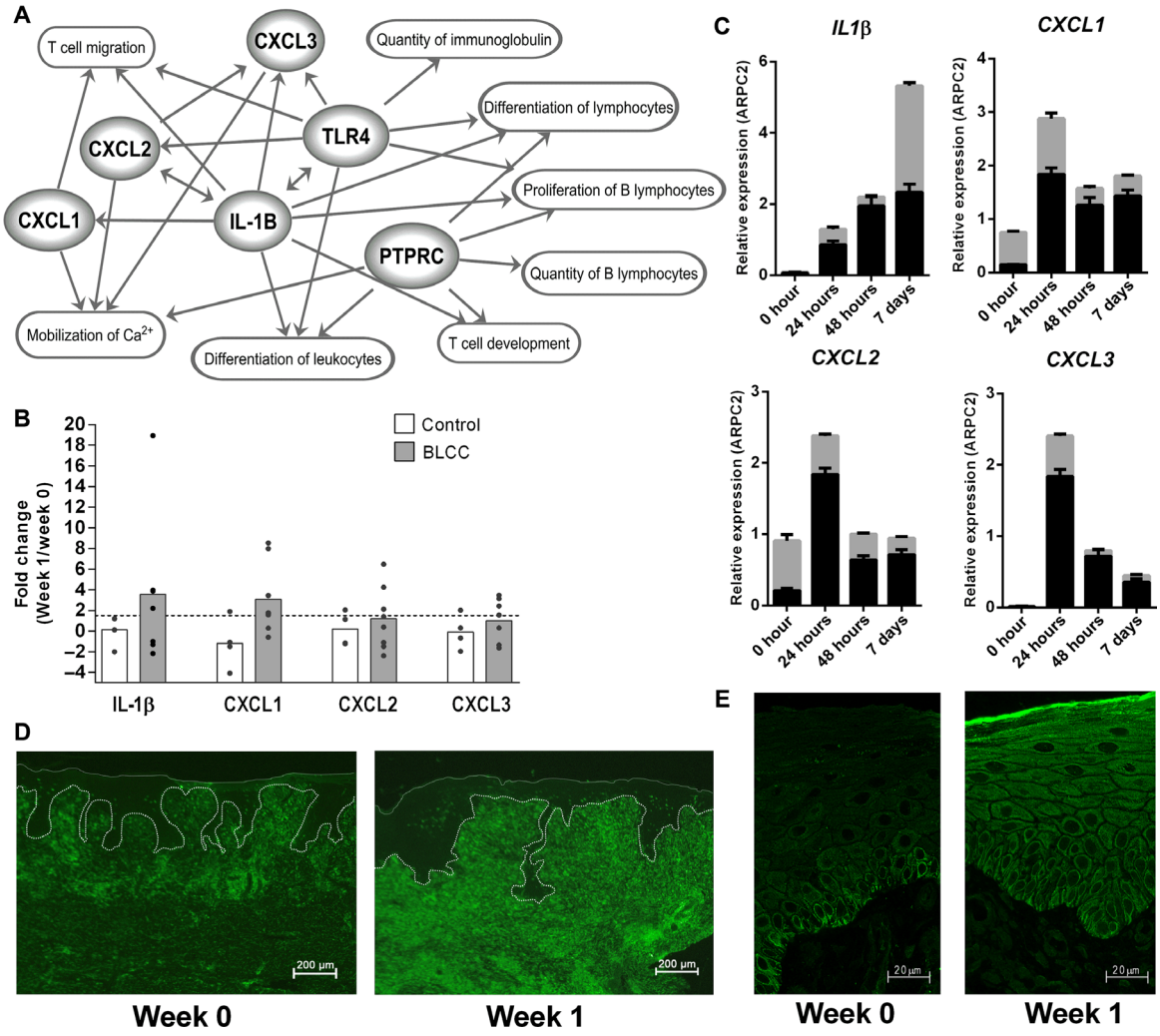

Fig. 6. Recapitulation of immune features of the acute wound healing phenotype in chronic VLUs after BLCC treatment. (**A**) Interplay of genes and biological functions jointly enriched in acute wounds ($n = 6$) and in BLCC-treated VLUs ($n = 6$). Links (arrows) are based on IPA-categorized literature findings. (**B**) qPCR expression of *IL1*β, *CXCL1*, *CXCL2*, and *CXCL3* in nonhealing VLUs 1 week after standard of care compression therapy (control, $n = 4$; unshaded bars) or compression therapy plus BLCC ($n = 8$; shaded bars). Dots represent fold expression change in individual paired samples 1 week after treatment, with bar height at the group mean. See also fig. S4. (**C**) *IL1*β, *CXCL1*, *CXCL2*, and *CXCL3* expression in an ex vivo model of acute wound healing in skin from $n = 2$ healthy donors, as determined by qPCR at 0, 1, 2, and 7 days after wounding. Gray and black bars represent expression in individual donors (means ± SEM of $n = 3$ technical replicates). (**D**) *PTPRC*-encoded CD45 receptor immunofluorescence staining of wound edge sections before (week 0) and after (week 1) BLCC treatment. (**E**) Immunofluorescence staining of wound edge sections for epidermal TLR4 expression before (week 0) and after (week 1) BLCC treatment. Images in (D) and (E) are representative of $n = 5$ study subjects before and after BLCC application (week 0 versus week 1).

level of individual genes as well as at the level of coordinately enriched pathways and biologic processes in acute wounds and BLCC-treated chronic ulcers. Their expression in healing versus nonhealing wounds might thus reflect fine-tuning of the adaptive immune response. Moreover, our data may indicate that prolonged inflammation present in nonhealing VLUs does not facilitate progression of healing found in acute wounds. Further, our data support the hypothesis that BLCC application reverts this suboptimal inflammation to activate acute inflammatory signaling similar to that seen in an acute wound healing environment.

The gene expression changes in paired VLU biopsies before and after treatment illustrated that BLCC triggered therapeutic reprogramming, reversing the nonhealing phenotype and activating prohealing pathways. These changes were not present in the control VLU group treated with standard of care compression therapy alone, indicating BLCC-specific effects. The only exception was *RARRES1* (retinoic acid receptor responder 1), an acute wound healing gene that was upregulated in both BLCC and control groups 1 week after treatment, perhaps reflecting continued optimal standard of care compression therapy, which both groups received, rather than a BLCC-unique mechanism. There are three possible mechanisms by which BLCC-initiated activation of healing may occur: (i) as demonstrated in vitro (*8*–*10*), the BLCC secretes growth factors and cytokines common to the prohealing pathways; (ii) the BLCC stimulates patients' VLU cells to activate prohealing signaling pathways; or (iii) the BLCC both secretes growth factors and activates prohealing signaling pathways within the VLUs. Although it is not possible to test which of these three mechanisms occurred in our study, our gene expression

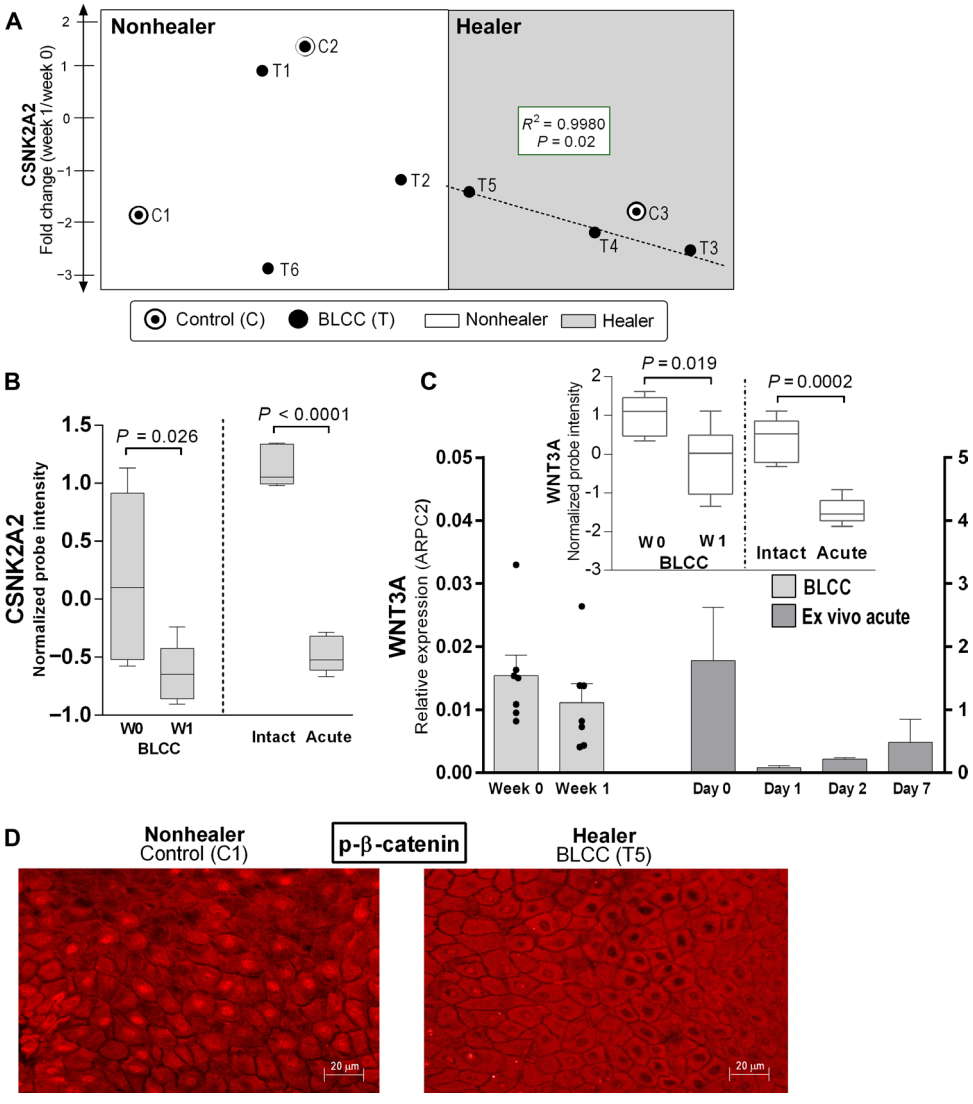

Fig. 7. Wnt/β-catenin signaling pathway in BLCC-treated VLUs and correlation with healing trajectory. (**A**) *CSNK2A2* expression changes and correlation with wound closure trajectory slope in three BLCC-responsive healers (shaded area, dashed line) compared to three nonhealers and three controls. See also fig. S5. (**B**) *CSNK2A2* expression in healing acute wounds (n = 6) and in chronic VLUs treated with BLCC (n = 6), as represented by box-and-whisker plots of microarray probe intensity (paired t test). (**C**) Relative *WNT3* expression in chronic VLUs (n = 7) before and after BLCC treatment and in acute wound healing (n = 2 healthy donor skin, ex vivo assay). Bars represent mean and SEM of three technical replicates after normalization to *ARPC2* internal control. Inset: *WNT3* microarray probe expression in paired biopsies of chronic VLUs before versus after BLCC treatment (n = 6), as well as acute wounds before versus 3 days after wounding (n = 6) (paired t test, $P < 0.05$). (**D**) Immunofluorescence of phosphorylated β-catenin (p-β-catenin) cellular localization in keratinocytes at the wound edge of a nonhealer VLU treated with standard of care (control) versus a BLCC-treated healer VLU. Images are representative of three controls and three BLCC-treated VLU healers.

data confirm that these important wound healing signaling pathways are activated and successfully executed in VLUs only upon BLCC and not standard compression treatment. Similarly, our findings do not pinpoint the exact cellular source(s) of the acute wound healing signals captured by our post-BLCC microarrays because there are several cell types that could contribute to the gene expression changes detected in the VLU edge after BLCC. However, because no BLCC DNA material was present in the VLUs after 1 week of treatment, our data indicate that the resident chronic VLU cells altered their transcriptomes in response to BLCC application.

Our study design was limited by the lack of repeated sampling and profiling of BLCC-treated VLUs at later time points. However, consistent with previous reports (*24*), we note again that no detectable BLCC cellular DNA remained in any of the VLU edge biopsies after 1 week of treatment. This observation implies that any direct BLCC-triggered effects are more likely to be detected within the initial week of treatment, which was the interval captured by our study, rather than at later time points. Given that original clinical trials of the BLCC's efficacy in VLUs only demonstrated a healing response months (rather than weeks) after treatment (*6*), the BLCC's early in vivo disappearance suggests that its initial therapeutic activity must be of sufficient intensity to effect sustained changes in the resident cells of the chronic wound, which then go on to gradually orchestrate successful wound closure.

The drawbacks of our study highlight a question that is of great importance to the field of chronic wound healing: should one perform in vivo "mechanism of action" studies in humans knowing that direct demonstration of causality cannot be attributed to anything more precise than the tissue response to the presence or absence of a particular therapeutic intervention? More to the point, the field of chronic wound healing is challenged by a lack of validated preclinical models, a factor that severely impedes translation of therapies to clinical practice. Given this reality, we believe that there are two choices: to continue using products without attempting to elucidate their mechanisms of action in this complex multifactorial disease and thus impede development of second-generation products for chronic wounds, or to accept the limitations of more descriptive, less mechanistic approaches to deciphering how patients' tissues respond to therapies to gain potential valuable clinical insights.

We argue that our clinical trial findings have direct clinical relevance. We were able to provide initial validation for specific biologic processes through which placement of the BLCC converts chronic nonhealing VLUs into an acute wound healing phenotype. These biologic processes may serve as specific targets and testable end points in the design and testing of future VLU therapies, which are sorely needed in the clinical setting. Moreover, we identified early changes in gene expression that, upon further validation in larger patient groups, may be predictive of healing outcomes after BLCC

application. These early indicator genes could help identify subsets of patients with chronic wounds who are most likely to derive clinical benefit from BLCC therapy. For instance, Wnt/β-catenin signaling is highly dysregulated in chronic VLUs (*2*, *3*), and we found that expression of the Wnt family member *CSNK2A2* correlated with healing in BLCC-treated ulcers. *CSNK2A2* might thus serve as a molecular marker that indicates commencement of healing with an initial application of the BLCC, warranting repeated treatments.

Fifteen years after initial pivotal clinical trials demonstrated efficacy of BLCC in healing chronic VLUs, data from our study provide new insights into how and why this occurs. We believe that similar studies can be integrated into clinical trials, providing new foundations upon which existing and novel diagnostic and therapeutic approaches for chronic wound healing can be examined. This has the potential to positively affect the lives of patients suffering from nonhealing VLUs.

MATERIALS AND METHODS

Study design

Study participants (ClinicalTrial.gov; NCT01327937) were recruited from patients presenting to the wound clinic at the University of Miami (Miami, FL) with VLUs. Written informed consent was obtained from all subjects enrolled in the clinical trial, and the study protocol and informed consent were approved by the Institutional Review Board (IRB) of the University of Miami. All subjects (n = 30), from screening (day −28) through day 0 visit, received the standard of care, including a dressing regimen of a foam dressing and a four-layered compression bandage system. Participants with noninfected target ulcers of >5 cm^2 that had not reduced in area by >40% during the 4-week screening period were randomized to either the control group receiving standard of care compression therapy (foam dressing plus four-layered compression bandage system, changed weekly by the investigator) (n = 9) or to the treatment group receiving weekly BLCC applications (Apligraf, Organogenesis Inc.), along with standard of care compression therapy (n = 15). Before all applications, the BLCC was fenestrated in a standardized manner using a #11 blade with six fenestrations per 44 cm^2. Skin biopsy specimens were obtained from the nonhealing edges of VLUs at the time of randomization (week 0) as well as 1 week later (week 1). Specimens were clinically designated by a physician as the most proximal skin edge to the ulcer bed. All patients were debrided, and local lidocaine injection was used for anesthesia. After week 5, all patients were monitored in the wound clinic for 12 weeks or until wound closure was achieved. In addition, to confirm the acute wound profile, ex vivo human skin experiments were performed on discarded human skin tissue obtained from voluntary surgeries (n = 2 donors) at the University of Miami Hospital and were found to be exempt under 45 CFR46.101.2 by the IRB at the University Of Miami Miller School Of Medicine. See the Supplementary Materials and Methods for full clinical study protocol and ex vivo experiment details.

Sample processing

Skin biopsies were processed as follows: samples were (i) embedded in OCT compound (Fisher Scientific), (ii) stored in formalin for paraffin embedding, and/or (iii) stored in RNA later or homogenized in TRIzol (Ambion/Applied Biosystems) or snap-frozen for subsequent RNA/protein isolation. Tissue morphology was evaluated using hematoxylin and eosin staining. Genotyping of a portion of each week 1 biopsy in the BLCC treatment group was performed by an outside laboratory (Esoterix Clinical Trials Services).

VLU morphology assessment

Biopsies obtained from VLU wound edges were formalin-fixed and paraffin-embedded. Sections (5 μm thick) were stained using hematoxylin and eosin following standard protocol and assessed for the presence of epidermis and dermis, thus confirming characteristic VLUs morphology as previously described (*2*).

Gene expression microarrays

Microarray experiments were performed at the University of Miami's Genomic Facility Core. RNA was amplified, fragmented, and hybridized to arrays using the GeneChip 3′ IVT Express kits following the manufacturer's protocol (Affymetrix). Total RNA (100 ng) was used as input for the Ambion WT Expression Kit to produce labeled single-stranded complementary DNA (cDNA) according to the manufacturer's instructions. Labeled products were hybridized to GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix). The staining, washing, and scanning of the arrays were carried out using Fluidics 450 station, GeneChip Operating Software, and GeneChip Scanner 3000 7G (Affymetrix). For quality control, Bioanalyzer analysis after the generation of complementary RNA, cDNA, and fragmentation was carried out using the 6000 Nano Kit (Agilent). Microarray data were analyzed as described in the Supplementary Materials and Methods.

Pathway analysis

Gene set enrichment analysis was performed using the Gene Ontology tool contained within the GeneSpring 13.0 Suite. Further pathway analysis and downstream target/functional predictions were performed using IPA (Qiagen; www.ingenuity.com). Microarray probes were mapped to the corresponding genes using the Ingenuity software; if multiple probes mapped to the same gene, the gene was used only once in enrichment calculations. Counts and lists of genes used as inputs for pathway analysis are found in tables S3 and S6. Statistical tools within the IPA software package used Fisher's exact test to detect the reported significantly enriched pathways, biologic processes, and upstream regulators; in all cases, enrichment P values were Benjamini-Hochberg–corrected for multiple testing.

Ex vivo acute wound model

Skin from two independent healthy donors was wounded and processed as described (*19*). RNA and formalin-fixed/paraffin-embedded sections were used for qPCR analyses. Refer to the Supplementary Materials and Methods for full details of experimental design and donor demographics.

Statistical analysis

Statistics for microarray data were performed as described in the Supplementary Materials and Methods. For qPCR validation studies, technical triplicates were included and groups were compared using two-sided paired t test. Correlation of nonparametric data was assessed using Spearman's test. Gene ontology enrichment P values were calculated within the GeneSpring 13.0 software package, which uses the Broad Institute GSEA algorithms. Pathway enrichment statistics were calculated within the Ingenuity software package using Fisher's exact test with Benjamini-Hochberg correction for multiple testing. Growth factor upstream regulator overlap P values were similarly calculated within IPA using Fisher's exact test. Two-sided testing was

performed with $\alpha < 0.05$ for all reported analyses, as specified in the corresponding figure legends.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/9/371/eaaf8611/DC1
Materials and Methods
Fig. S1. Expression heat maps of top BLCC-regulated genes in clinical trial subjects.
Fig. S2. Networks of growth factors and their known effects on gene targets that are also regulated in chronic VLUs after BLCC application.
Fig. S3. Clinical trajectories of VLU study subjects.
Fig. S4. Subject-specific expression of cytokines.
Fig. S5. Posttreatment healing trajectories.
Table S1. Subject disposition.
Table S2. Exclusions from analysis of primary outcome.
Table S3. Significantly regulated entities in the BLCC and control treatment groups.
Table S4. Genes corresponding to the top 10 enriched pathways after BLCC treatment.
Table S5. Evidence for TGFB1 activation post-BLCC treatment.
Table S6. Establishment of reference gene expression profiles for acute and chronic VLU wound healing.
Table S7. Significantly regulated entities in the acute and chronic wound reference profiles.
Table S8. Regulated genes corresponding to enriched pathways shared by acute wounds and BLCC-treated VLUs.
Table S9. Regulated genes corresponding to enriched biological processes shared by acute wounds and BLCC-treated VLUs.
Table S10. Genes correlating with the healing trajectory in VLUs after BLCC treatment.

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A bioengineered living cell construct activates an acute wound healing response in venous leg ulcers

Rivka C. Stone, Olivera Stojadinovic, Ashley M. Rosa, Horacio A. Ramirez, Evangelos Badiavas, Miroslav Blumenberg and Marjana Tomic-Canic (January 4, 2017)

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Editor's Summary

Activating healing in chronic wounds

Effective therapies for chronic venous leg ulcers (VLUs) remain elusive, in part, because of the incomplete understanding of the pathophysiology of nonhealing wounds. Stone *et al*. conducted a postmarket clinical trial using transcriptomics to understand the mechanisms of action of an FDA-approved bilayered living cellular construct (BLCC) in nonhealing VLUs. After 1 week of BLCC treatment in addition to standard of care compression therapy, nonhealing VLUs showed changes in inflammation and gene expression characteristic of acutely healing wounds. This study provides mechanistic insight into how the acute healing process can be activated by a cell therapy in chronic nonhealing wounds.

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