Wound Healing Response for Bi-layered Living Cellular Construct Promotes M2 Macrophage Phenotype

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ABSTRACT

Bi-layered living cellular construct (BLCC†) is the only bioengineered cellular skin substitute approved by the FDA to heal DFUs and VLUs. It consists of an epidermal layer containing keratinocytes and a dermal layer consisting of fibroblasts, mimicking human skin. Fenestration, or wounding of the BLCC, results in a wound healing response of the fenestration *in vitro*, suggesting that BLCCs retain advanced capacity for healing. Building upon previous MOA studies, we hypothesized that signals generated by BLCCs not only impact the construct's ability to heal itself, but also provide signals to the patient's wound bed, modulating the local immune and inflammatory responses.

4mm biopsy punches of BLCCs (wounds) were placed onto a tissue engineered dermal equivalent layer (DE). Wounds were allowed to heal and were assessed on days 3, 6, and 9 for epithelial outgrowth, genetic regulation, and protein generation. THP-1 polarized macrophages were treated with conditioned media from the healing BLCCs and assessed for promotion of either M1 or M2 phenotypes as well as cellular migration.

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EXPERIMENTAL MODEL

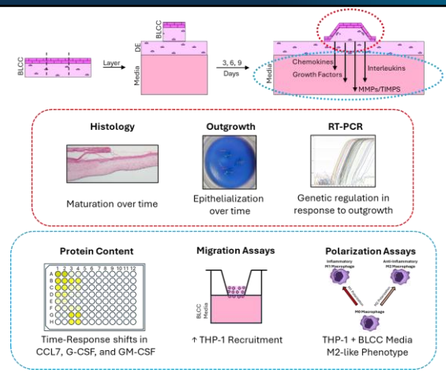

Figure 1. Experimental model. BLCC biopsy punches were placed onto dermal equivalent (DE) layers and allowed to grow for 3, 6, or 9 days. At each time point, designated tissue samples were assessed (red circle), and media was collected (blue circle). Media was assessed for proteins generated by BLCC outgrowth and their impact on monocyte/macrophage (THP-1) recruitment and activation.

BLCC RE-EPITHELIALIZES IN RESPONSE TO WOUNDING

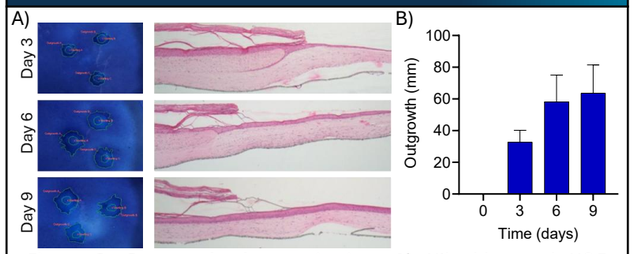

Figure 2. Outgrowth characterization. A) Nile blue and H&E staining of outgrowth. **B)** Area of outgrowth. Means ± StDev.

OUTGROWTH OF BLCC DRIVEN BY GENETIC REGULATION

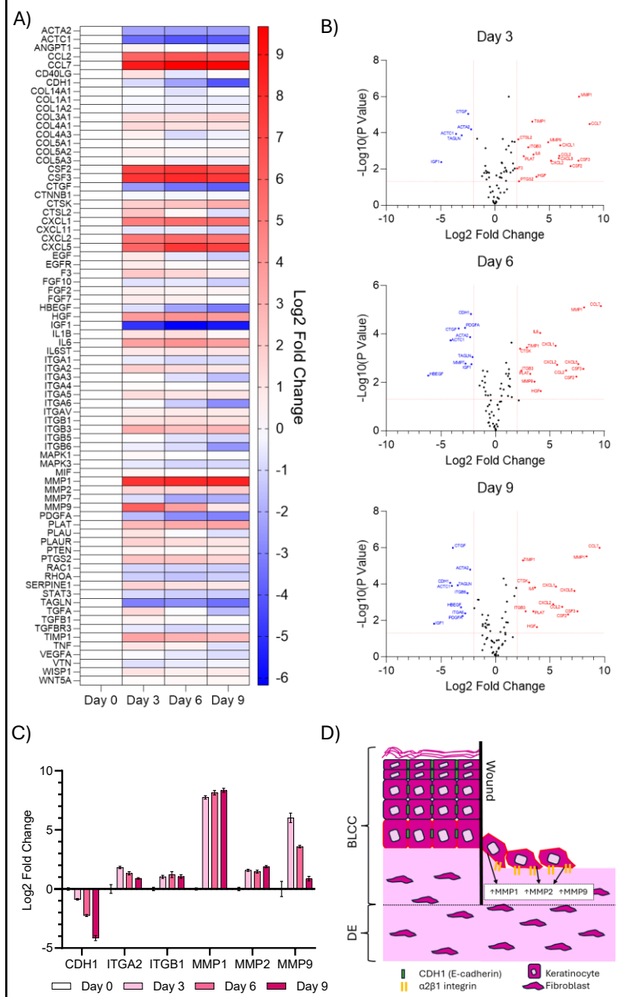

Figure 3. Genes up/down regulated as BLCC extends onto DE layer. A) Heatmap showing genes up/downregulated; Log2 fold change expression. **B)** Volcano plots with threshold cutoffs at P values ≤ 0.05 and ± 2 Log2 fold changes. Upregulated genes shown in red, downregulated genes shown in blue. **C)** Specific genes of interest involved in EMT of keratinocytes in response to wounding. **D)** Schematic of keratinocyte migration during re-epithelialization. Basal keratinocytes lose E-cadherin expression and upregulate genes for the two subunits of α2β1 integrin to migrate across the collagen matrix. Binding of α2β1 upregulates MMP expression, specifically MMP1, with over a 100-fold increase in expression. Data expressed as means ± standard deviation.

GENE ONTOLOGY HIGHLIGHTS IMPACT ON IMMUNE RESPONSE

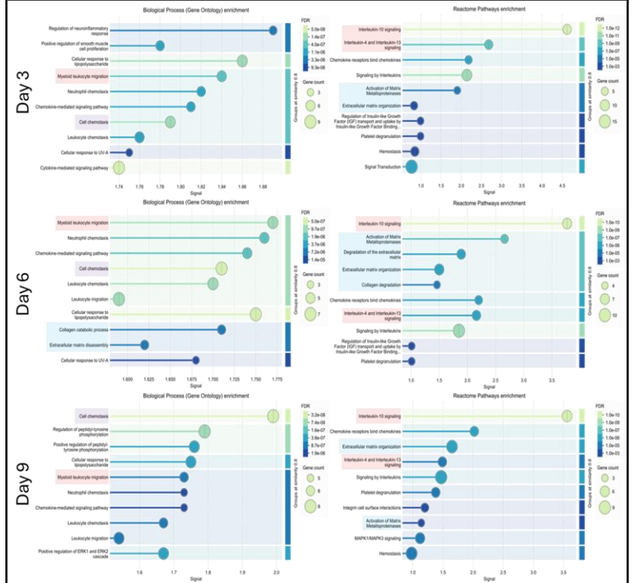

Figure 4. Gene ontology assessment identifies pathways associated with healing and immune recruitment. Gene ontology and reactome pathway assessment from statistically relevant genes identified in **Figure 3B**. Red boxes = myeloid pathways, blue boxes = remodeling pathways, and purple boxes = cell migration.

WOUNDED BLCC PROMOTES MONOCYTE RECRUITMENT

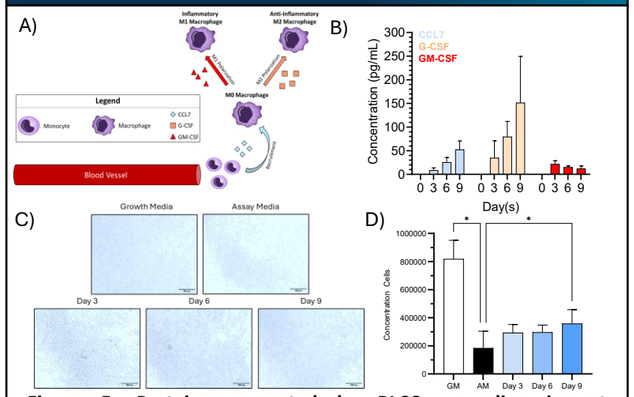

Figure 5. Proteins generated by BLCC wounding impact recruitment. A) Proposed immune modulation MOA. **B)** Protein concentration for recruitment of monocytes (CCL7), M2 polarization (G-CSF/CSF2), and M1 polarization (GM-CSF/CSF3). Data presented as Means ± StDev. **C)** Qualitative or **D)** quantitative assessment of migration to growth media (GM), assay media (AM), or conditioned media. Means ± standard deviation. * p ≤ 0.05

WOUNDED BLCC DRIVES AN M2-LIKE MACROPHAGE PHENOTYPE

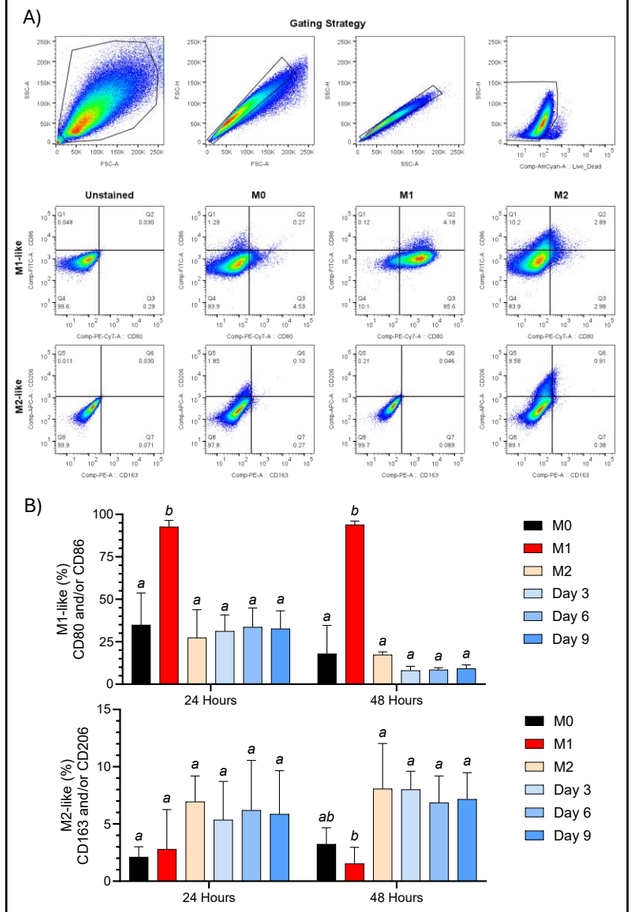

Figure 6. Conditioned media promotes an M2-like phenotype. A) Gating strategy for M1-like (CD80 and/or CD86) or M2-like (CD163 and/or CD206). **B)** THP-1 cells were stimulated with PMA (M0), then polarized to M1 or M2 macrophages using IFN-γ/LPS (M1), IL-4/IL-13 (M2), or conditioned media (25%) from wounded BLCCs on days 3, 6, or 9. Cells stimulated with conditioned media are statistically more M2-like. Compact letter display statistical groupings with statistical difference set to p ≤ 0.05.

CONCLUSIONS

1) Wounding of BLCC triggers an EMT phenotype shift, resulting in keratinocyte migration and epithelialization.
2) Conditioned media from wounded BLCCs promotes migration and activation of an M2-like phenotype,

<u>Treatment with BLCC recruits immune effector cells and provides a microenvironment to promote a wound healing phenotype.</u>

ORGANOGENESIS