Lyophilized Human Amnion Chorion Membrane Allografts Modulate Reactive Oxygen Species Production *in vitro*

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INTRODUCTION

The maternal-fetal interface is a unique immunomodulatory microenvironment in mammals and may be leveraged therapeutically in wound healing through the application of amniotic tissue allografts. Macrophages are crucial in wound healing for orchestrating the shift from inflammation to tissue repair by transitioning from a pro-inflammatory to a pro-repair phenotype. The release of regulatory growth factors and signaling molecules by various macrophage phenotypes directs proper progression through each healing stage and timely wound closure. Conversely, inflammatory dysregulation in macrophages is patho-mechanically linked to chronic inflammation, impaired cellular functionality, and stalled wound healing (1). The underlying mechanism is multi-factorial, but often attributed to increased inflammasome activation, excessive reactive oxygen species (ROS) production and ineffective clearance of cellular debris resulting in a self-reinforcing state of inflammation (2). This study aimed to evaluate the effect of tri-layer lyophilized human amnion chorion membrane (LHACM*) on macrophage redox state, phenotypic modulation, and functional differences in response to inflammatory stimulus *in vitro*.

MATERIALS AND METHODS

Eluate Preparation: Human amniotic tissue (amnion, intermediate, and chorion layers) was processed using the Proprietary ‡PURION® process, including cleansing followed by lyophilization and terminal sterilization to produce LHACM. Soluble factors from LHACM were extracted in assay-appropriate complete media at 4°C for 16 hours.

Cell Culture: Human monocytic THP-1 cells (ATCC TIB-202) and human T lymphoblast Jurkat cells (ATCC TIB-152) were maintained in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. THP-1 monocytes were differentiated into resting macrophages (M0) using 10 ng/mL phorbol 12-myristate 13-acetate (PMA). M0 macrophages were incubated with 20, 10 and 1 mg/mL LHACM for 48 hours to produce LHACM-elicited macrophages for downstream use in the efferocytosis assay.

Pro-inflammatory Response Measurement: To simulate pro-inflammatory polarizing conditions, M0 macrophages were stimulated with 100 ng/mL LPS and 20 ng/mL interferon gamma (IFNγ) in the presence or absence of 1, 10, or 20 mg/mL LHACM eluate for 24 hours. Macrophage culture supernatants were harvested post-stimulation, and assays to measure soluble pro-inflammatory protein concentrations (Luminex) and Caspase-1-mediated inflammasome activity (Promega) were performed in accordance with the manufacturer's instructions.

Luminex Assay: Culture media was collected from M0 THP-1 cells that were stimulated for 24 hours with 100 ng/mL LPS and 20 ng/mL IFNγ, in the presence or absence of 20, 10 and 1 mg/mL LHACM extract. The media was assessed for pro-inflammatory cytokines using bead-based Luminex multiplex assay (R&D systems) in accordance with the manufacturer's instructions. Briefly, samples were incubated with beads coated in antibodies to analytes of interest. Samples were then incubated with biotinylated detection antibodies followed by a streptavidin-phycoerythrin (PE) reporter. Recovered targets were quantified using Luminex instrument (Luminex FLEXMAP 3D, R&D Systems).

Caspase-1-mediated Inflammasome Assay: Culture media was collected from M0 THP-1 cells that were stimulated for 24 hours with 100 ng/mL LPS and 20 ng/mL IFNγ, in the presence or absence of 20, 10 and 1 mg/mL LHACM extract and was assessed using a Caspase-Glo 1 Inflammasome Assay Kit (Promega). Proteolytic activity of Caspase-1 in the culture media was measured against a cleavable Luciferase-linked reagent using luminescence readings acquired on a Synergy Mx Microplate Reader (Biotek) according to the manufacturer's instructions.

ROS Evaluation: To evaluate ROS production in live cells, M0 macrophages were stimulated with 100 ng/mL LPS and 20 ng/mL IFNγ in the presence or absence of 20, 10, 5, and 1 mg/mL LHACM extract for 48 hours before being harvested. Single cell suspensions were stained with viability dye (SYTOX Red, ThermoFisher) and CellROX Green antibody (ThermoFisher), and data acquisition was performed using a 3-laser Cytek Northern Lights flow cytometer. Subsequent data analysis and graphing was performed using FlowJo (v10.10.0) and GraphPad Prism (v10.2.2) software.

Efferocytosis Assay: For measuring macrophage efferocytotic activity, untreated and LHACM-elicited M0 macrophages were incubated with CFSE-labeled apoptotic bait cells for 16 hours before being harvested and analyzed. Single cell suspensions were acquired using a 3-laser Cytek Northern Lights flow cytometer, and subsequent data analysis and graphing was performed using FlowJo (v10.10.0) and GraphPad Prism (v10.2.2) software.

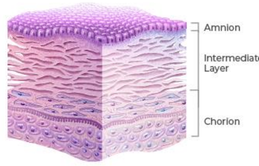

Figure 1. LHACM is a tri-layer allograft membrane. LHACM consists of human amnion, intermediate, and chorion placental layers. Placental tissue was prepared via the ‡PURION® process to generate terminally sterilized, lyophilized tri-layer graphs.

REFERENCES

1. Headland, S. E., & Norling, L. V. (2015). The resolution of inflammation: Principles and challenges. *Seminars in Immunology, 27*(3), 149–160. https://doi.org/10.1016/j.smim.2015.03.014
2. Krzyszczyk, P., Schloss, R., Palmer, A., & Berthiaume, F. (2018). The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes. *Frontiers in physiology*, 9, 419. https://doi.org/10.3389/fphys.2018.00419

RESULTS

LHACM modulates the macrophage pro-inflammatory response

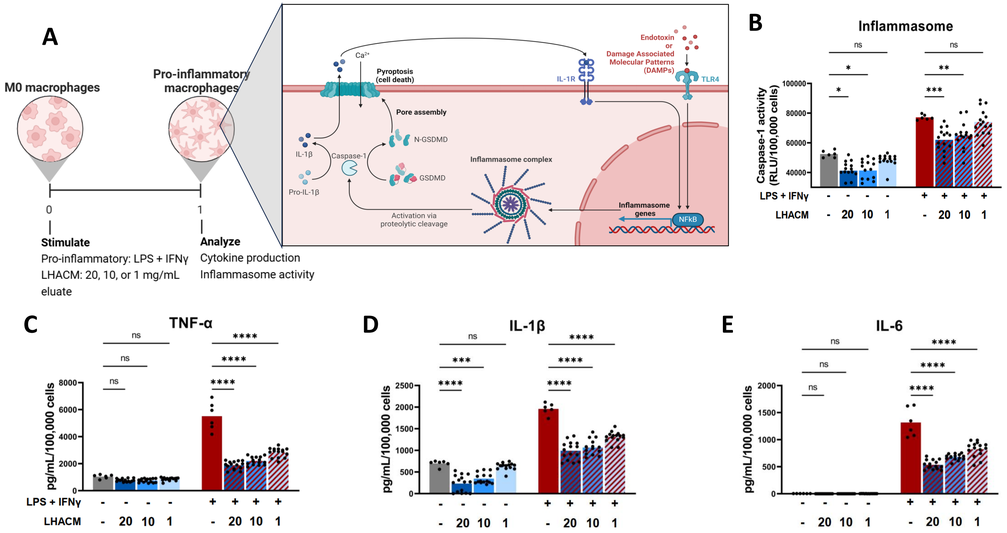

Figure 2. LHACM dampens the macrophage pro-inflammatory response. (A) To induce an inflammatory response and inflammasome activity, THP-1 M0 macrophages were stimulated for 24 hr with 100 ng/mL LPS and 20 ng/mL IFNγ in the presence or absence of 20, 10, or 1 mg/mL LHACM. **(B)** Inflammasome function was determined by measuring the proteolytic activity of Caspase-1 in the supernatant against a cleavable Luciferase-linked reagent. Pro-inflammatory cytokine production was measured following stimulation via Luminex analysis of cell supernatants; (C) TNF-α., (D) IL-1β, (E) IL-6. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 relative to control based on a one-way ANOVA with a Tukey test; ns: statistically insignificant. Summary graphic (A) was created using BioRender.

LHACM treatment increases macrophage efferocytosis

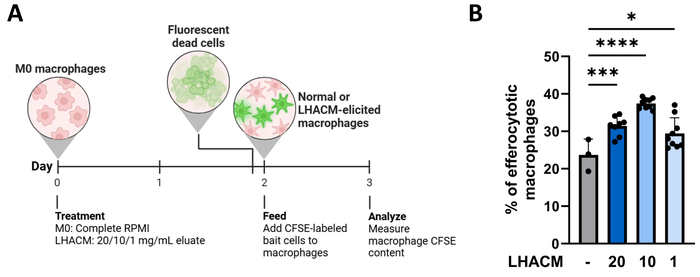

Figure 3. LHACM-elicited macrophages exhibit increased efferocytotic activity. (A) THP-1 M0 macrophages were treated with 20, 10, or 1 mg/mL LHACM for 48 hours before being washed and incubated with RPMI containing CFSE-labeled apoptotic bait cells. **(B)** The frequency of bait cell-containing macrophages was measured by flow cytometry. Data shown as mean ± SD, n=3 for M0 and n=3 for each of three donors for each treatment. *p<0.05, ***p<0.001, ****p<0.0001 relative to M0 based on a one-way ANOVA with Dunnett's test. Summary graphic (A) was created using BioRender.

All authors are employees of MIMEDX Group, Inc.

*EPIEFFECT®, MIMEDX Group, Inc. Marietta, GA; ‡PURION® Process, MIMEDX Group, Inc., Marietta GA

RESULTS

LHACM reduces ROS under pro-inflammatory polarizing conditions

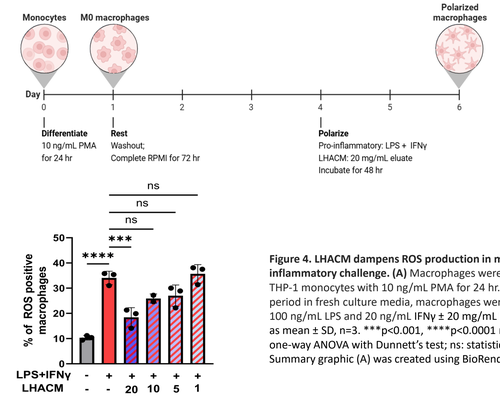

Figure 4. LHACM dampens ROS production in macrophages during pro-inflammatory challenge. (A) Macrophages were generated by treating THP-1 monocytes with 10 ng/mL PMA for 24 hr. Following a 72 hr rest period in fresh culture media, macrophages were polarized for 48 hr with 100 ng/mL LPS and 20 ng/mL IFNγ ± 20 mg/mL LHACM. Data represented as mean ± SD, n=3. ***p<0.001, ****p<0.0001 relative to M1 based on a one-way ANOVA with Dunnett's test; ns: statistically insignificant. Summary graphic (A) was created using BioRender.

GRAPHICAL OVERVIEW

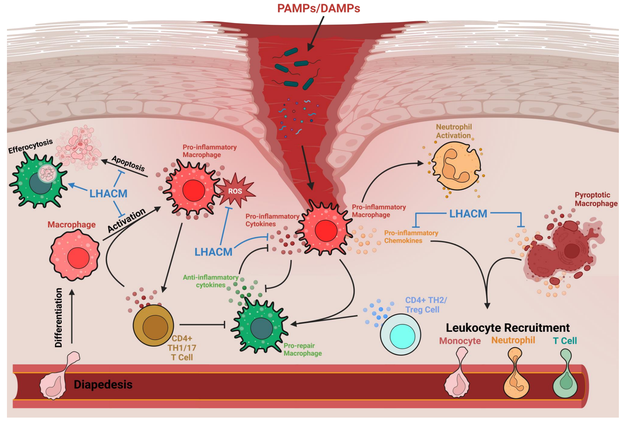

Figure 5. LHACM modulates multiple aspects of macrophage effector function. LHACM modifies macrophage biology both in the presence and absence of inflammatory stimulus by blunting pro-inflammatory polarization, regulating ROS production, promoting cellular debris clearance, and dampens inflammasome effector protein Caspase-1 activity. Created using BioRender.

CONCLUSION

The findings from this study suggest that LHACM attenuates macrophages' response to inflammatory stimuli *in vitro* while promoting pro-repair functionality. This research contributes valuable insights into the potential uses of LHACM to create an ideal wound healing microenvironment, emphasizing its role in regulating macrophage activity.