Lyophilized Human Amnion Chorion Membrane Allografts Modify Inflammatory Monocyte-derived Macrophage's Phenotype in vitro

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SAWC , April 2026

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 results in 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 sustained macrophage activation, excessive inflammatory cytokine production, and ineffective recruitment of cell types essential for inflammation resolution which results in a self-reinforcing state of inflammation (2). Primary monocyte-derived macrophages (MDM) offer an accessible and physiologically relevant model for studying human innate immune responses. This study aimed to evaluate the effect of tri-layer lyophilized human amnion chorion membrane (LHACM*) on primary monocyte-derived macrophages' biology 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 basal RPMI-1640 media at 4°C overnight. Eluate from three LHACM tissue donors was pooled together to reduce the burden of treatment variability.

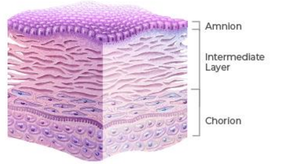

Figure 1. LHACM is a tri-layer allograft membrane. LHACM consists of human amnion, intermediate, and chorion placental layers. Placental tissue was prepared via the ‡PURION® process to generate terminally sterilized, lyophilized tri-layer graphs.

Cell Culture: Human peripheral blood mononuclear cells (PBMC) were isolated from leukocyte reduction system chambers using Ficoll-Paque density gradient centrifugation. Monocytes were isolated from PBMC samples using a MagniSort™ Human pan-Monocyte Enrichment Kit (Invitrogen) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, and 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) for six days to differentiate into resting state (M0) MDMs. M0 MDMs were detached using Accutase and plated at either at $5x10^4$ cells/well in 96-well culture plates or at $7.5x10^5$ cells/well in 12-well culture plates for subsequent polarization and treatment as appropriate per assay condition.

Inflammatory Response Measurement: To simulate pro-inflammatory polarizing conditions, M0 MDMs were stimulated with 100 ng/mL LPS and 20 ng/mL interferon gamma (IFNγ) in the absence (M1) or presence of 20 or 10 mg/mL LHACM eluate for 24 hours. Pro-repair macrophage controls were induced by treating M0 MDMs with 20 ng/mL IL-4 and IL-13 (M2a) or 20 ng/mL IL-10 (M2c) for 24 hours.

Gene Expression Analysis by qRT-PCR: To evaluate transcriptional changes, total RNA from three wells of each treatment group was extracted and pooled using a GeneJET RNA Purification Kit (Thermo Fisher Scientific). RNA quality and quantity were assessed using a Thermo Fisher Scientific NanoDrop 2000. cDNA was synthesized using a SuperScript IV VILO Kit with ezDNase (Thermo Fisher Scientific). Gene primers tested included *CD80*, *IL6*, *IL1B*, *TNF*, *CCL2*, and *NOS2* (Thermo Fisher Scientific). 18S rRNA Control (Thermo Fisher Scientific) was used as an internal control for relative gene expression analysis. Data acquisition was performed using a QuantStudio 7 Flex RT-PCR system (Thermo Fisher Scientific). Subsequent data analysis was performed using GraphPad Prism.

Luminex Assay: Culture media was collected from M0 MDM cells, M2a/M2c MDM controls, and MDMs that were stimulated for 24 hours with 100 ng/mL LPS and 20 ng/mL IFNγ, in the presence or absence of 20 or 10 mg/mL LHACM extract. The media was assessed for pro-inflammatory cytokines and pro-repair chemokines 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) and normalized to viable cells.

Flow Cytometry: MDM single cell suspensions were stained with viability dye (Zombie NIR, BioLegend) and antibodies (BioLegend) against key macrophage activation markers CD80, CD86, and HLA-DR. Data acquisition was performed using a 3-laser Cytek Northern Lights flow cytometer. Subsequent data analysis was performed using FlowJo software and GraphPad Prism.

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

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

All authors are employees of MIMEDX Group, Inc.

RESULTS

LHACM treatment blunts expression of pro-inflammatory activation surface proteins in MDMs

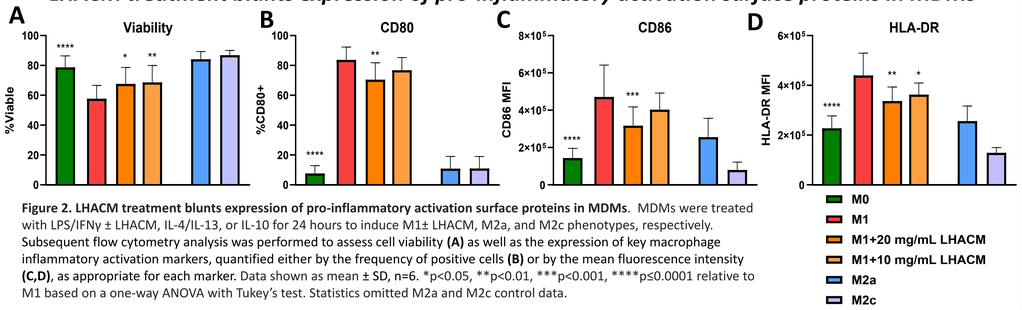

Figure 2. LHACM treatment blunts expression of pro-inflammatory activation surface proteins in MDMs. MDMs were treated with LPS/IFNγ ± LHACM, IL-4/IL-13, or IL-10 for 24 hours to induce M1± LHACM, M2a, and M2c phenotypes, respectively. Subsequent flow cytometry analysis was performed to assess cell viability **(A)** as well as the expression of key macrophage inflammatory activation markers, quantified either by the frequency of positive cells **(B)** or by the mean fluorescence intensity **(C,D)**, as appropriate for each marker. Data shown as mean ± SD, n=6. *p<0.05, **p<0.01, ***p<0.001, ****p≤0.0001 relative to M1 based on a one-way ANOVA with Tukey's test. Statistics omitted M2a and M2c control data.

LHACM modulates the signaling profile of MDMs in response to inflammatory stimulation

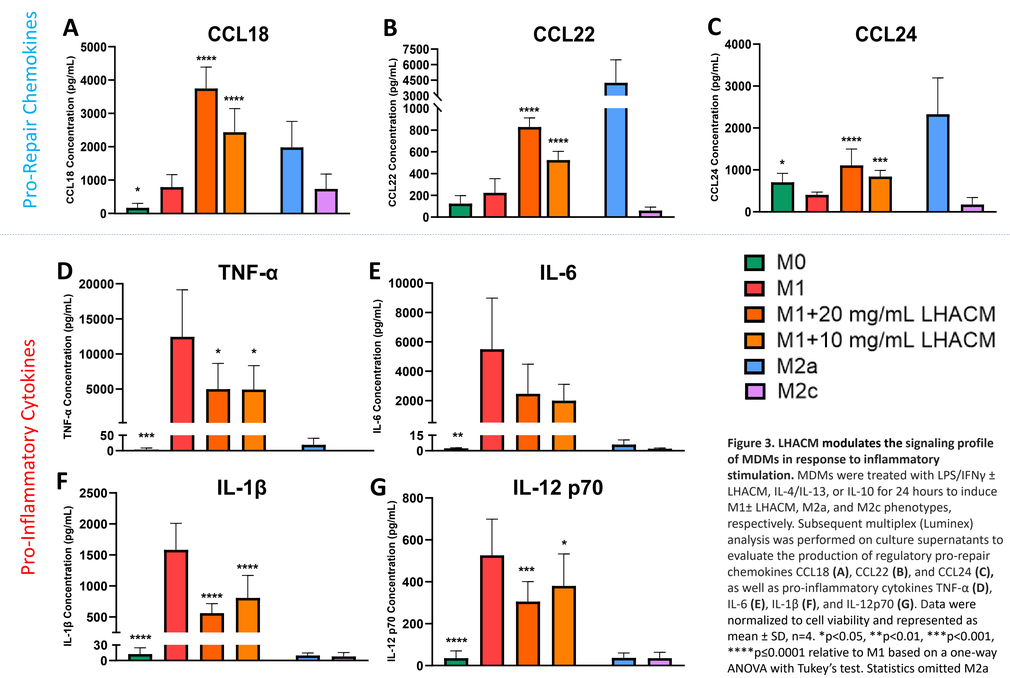

Figure 3. LHACM modulates the signaling profile of MDMs in response to inflammatory stimulation. MDMs were treated with LPS/IFNγ ± LHACM, IL-4/IL-13, or IL-10 for 24 hours to induce M1± LHACM, M2a, and M2c phenotypes, respectively. Subsequent multiplex (Luminex) analysis was performed on culture supernatants to evaluate the production of regulatory pro-repair chemokines CCL18 **(A)**, CCL22 **(B)**, and CCL24 **(C)**, as well as pro-inflammatory cytokines TNF-α **(D)**, IL-6 **(E)**, IL-1β **(F)**, and IL-12p70 **(G)**. Data were normalized to cell viability and represented as mean ± SD, n=4. *p<0.05, **p<0.01, ***p<0.001, ****p≤0.0001 relative to M1 based on a one-way ANOVA with Tukey's test. Statistics omitted M2a and M2c control data.

RESULTS

LHACM treatment modulates M1-associated gene expression in pro-inflammatory MDMs

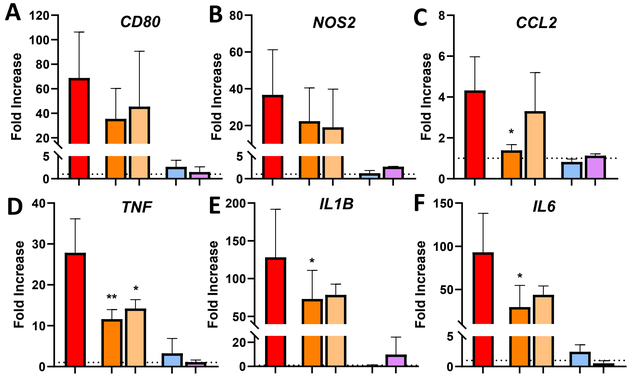

Figure 4. LHACM treatment moderates M1-associated gene expression in pro-inflammatory MDMs. MDMs were treated with LPS/IFNγ ± LHACM, IL-4/IL-13, or IL-10 for 24 hours to induce M1± LHACM, M2a, and M2c phenotypes, respectively. Subsequent cDNA was generated and real-time PCR was performed to assess fold change in gene expression for *CD80* **(A)**, *NOS2* **(B)**, *CCL2* **(C)**, *TNF* **(D)**, *IL1B* **(E)**, and *IL6* **(F)**. Data shown as mean ± SD, n=3 relative to M0 control. *p<0.05, **p<0.01 relative to M1 based on a one-way ANOVA with Tukey's test. Statistics omitted M2a and M2c control data.

GRAPHICAL OVERVIEW AND CONCLUSION

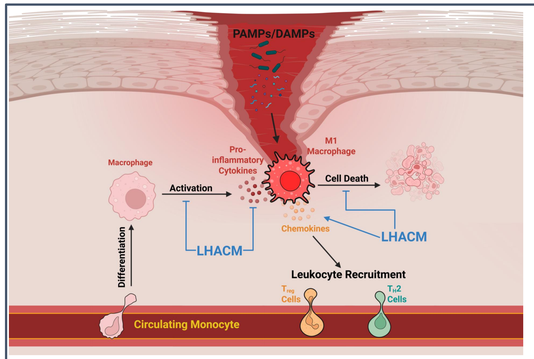

Figure 5. LHACM modulates multiple levels of macrophage biology. LHACM modifies macrophage biology in the presence of inflammatory stimuli by affecting pro-inflammatory activation, pro-inflammatory cytokine production, pro-repair chemokine production, and cell survival. Graphic was created using BioRender.

The findings from this study suggest that LHACM attenuates macrophages' pro-inflammatory response to inflammatory stimuli at multiple biological levels while supporting pro-repair chemokine production associated with effective wound healing *in vitro*. This research contributes valuable insights into the potential uses of LHACM to create an ideal wound healing microenvironment, emphasizing its role in regulating macrophage biology.