



Decellularized amniotic membrane promotes the anti-inflammatory response of macrophages *via* PI3K/AKT/HIF-1 α pathway

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ABSTRACT

Macrophages undergo dynamic transitions between M1 and M2 states, exerting profound influences on both inflammatory and regenerative processes. The biocompatible and wound-healing properties of decellularized amniotic membrane (dAM) make it a subject of exploration for its potential impact on the anti-inflammatory response of macrophages. Experimental findings unequivocally demonstrate that dAM promotes anti-inflammatory M2 polarization of macrophage, with its cytokine-rich content posited as a potential mediator. The application of RNA sequencing unveils differential gene expression, implicating the hypoxia inducible factor-1 α (HIF-1 α) signaling pathway in this intricate interplay. Subsequent investigation further demonstrates that dAM facilitates anti-inflammatory M2 polarization of macrophage through the upregulation of epidermal growth factor (EGF), which, in turn, activates the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway and stabilizes HIF-1 α . This cascade results in a noteworthy augmentation of anti-inflammatory gene expression. This study significantly contributes to advancing our comprehension of dAM's immunomodulatory role in tissue repair, thereby suggesting promising therapeutic potential.

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Macrophages, a crucial type of immune cell, possess the ability to polarize into distinct states, namely M1 and M2, in response to various stimuli and environmental conditions [1]. The process of macrophage polarization plays a significant regulatory role in tissue repair and regeneration. During both acute and chronic inflammation, M1 macrophages can generate pro-inflammatory factors, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and IL-6, along with a range of oxidases, including nitric oxide (NO), to facilitate antimicrobial activity and sterilization. This inflammatory response aids in the elimination of pathogens and tissue damage, thereby promoting the initiation of the repair process [2]. Subsequently, as the inflammation subsides, factors released by M2 macrophages, such as IL-4, IL-10, and transforming growth factor- β (TGF- β), foster cell proliferation, anti-inflammatory responses, anti-fibrotic mechanisms, and angiogenesis, driving tissue repair and regeneration while mitigating excessive inflammatory reactions arising from tissue damage [3]. Moreover, M2 macrophages are capable of restraining excessive fibrotic reactions in the aftermath of inflammation. They achieve this by secreting

fibrolytic enzymes, including matrix metalloproteinases (MMPs), which degrade the excessive deposition of collagen during fibrosis. Depending on external stimuli and the stage of tissue repair, macrophages play a pivotal role in the eradication of inflammation and the facilitation of tissue repair processes, thus promoting the resolution of inflammation and the regeneration of tissue [4]. Consequently, comprehensive investigation into the mechanisms governing macrophage polarization and the regulation of their polarization states holds the potential to offer novel therapeutic strategies and approaches for tissue repair and disease treatment.

Hydrogels have been frequently used to repair tissues after injury because they can provide a moist environment, simulate the extracellular matrix. However, it does not contain any active cytokines by itself and must be combined with other cytokines to work well [5–7]. Decellularized amniotic membrane (dAM) is a natural material extensively utilized in the field of biomaterials due to its wide range of unique properties [8]. Firstly, it exhibits exceptional biocompatibility by eliminating the potential for cell immune reactions and rejection responses through the removal of cells. Secondly, it possesses the capacity to enhance wound healing and tissue regeneration. This is attributed to the presence of various bioactive molecules such as cytokines, growth factors, and collagen proteins within the amniotic mem-

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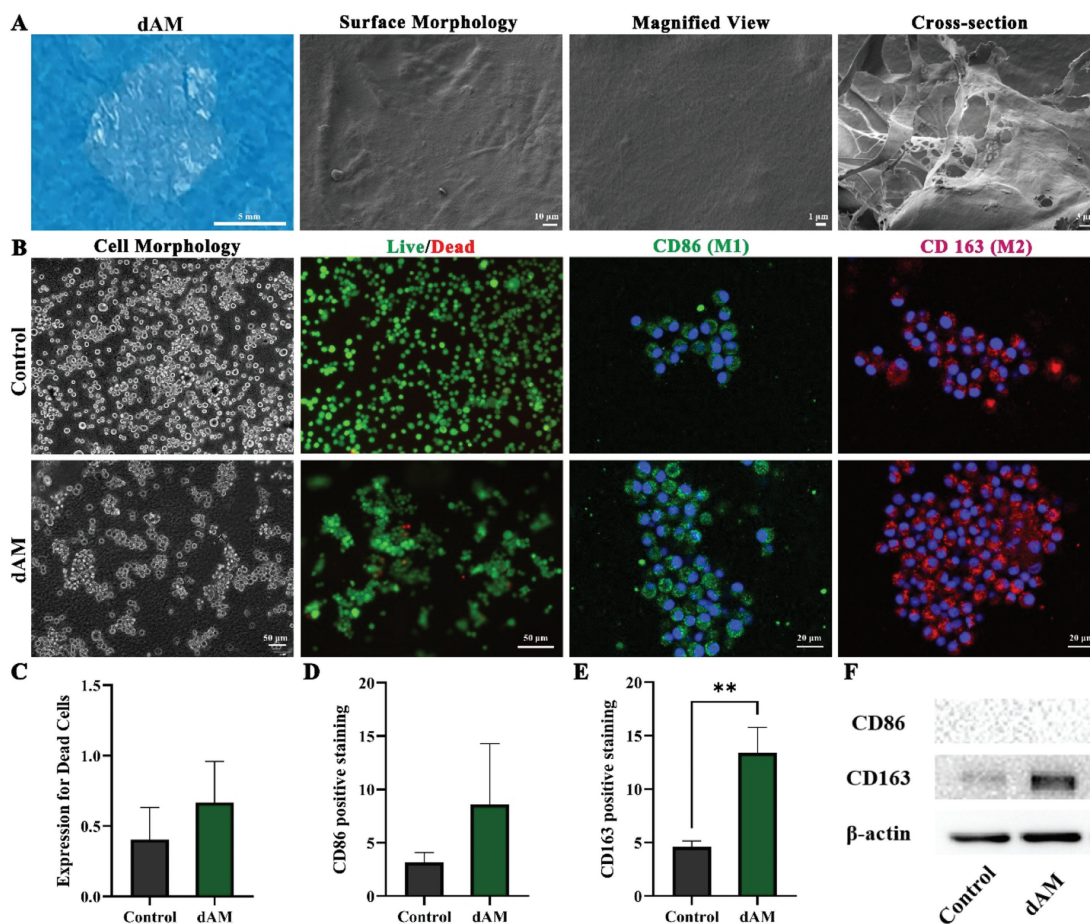


Fig. 1. The characteristics of dAM and its effects on macrophages. (A) General appearance and scanning electron microscopy (SEM) images of dAM. (B) Cell morphology and immunofluorescence images of live/dead (green and red), CD86 (green) and CD163 (red) for macrophage co-cultured with dAM. (C) Expression for dead cells for live/dead staining. (D) CD86 positive staining for immunofluorescence images. (E) CD163 positive staining for immunofluorescence images. Data are presented as mean \pm standard deviation (SD) ($n=3$). one-way ANOVA. $**P < 0.01$. (F) Western blot of CD86 and CD163 for macrophage co-cultured with dAM.

brane, which stimulate cell proliferation and differentiation, consequently facilitating tissue repair and regeneration [9]. Moreover, dAM demonstrates remarkable characteristics as a biological scaffold, closely resembling the structure of the natural extracellular matrix (ECM). This provides a three-dimensional environment that supports and guides cell growth. Additionally, it possesses antimicrobial and anti-inflammatory properties, attributed to the presence of bioactive molecules like antimicrobial peptides and inflammation-regulating factors, which effectively inhibit bacterial growth and inflammatory responses, promoting wound healing and preventing infection. Previous studies have shown that dAM can promote the anti-inflammatory response of macrophages for tissue repair and regeneration; however, the specific underlying molecular mechanisms remain unclear [10,11]. Therefore, the objective of this study is to preliminarily explore the molecular mechanisms through which dAM promotes the anti-inflammatory response of macrophages.

The dAM is thin and fragile, and electron microscopy reveals its porous 3D structure, which is characteristic of the ECM (Fig. 1A). Macrophages were cultured on the dAM to investigate its effect on macrophage polarization. NR8383 macrophages (iCell Bioscience Inc., China) were purchased and cultured in a macrophage culture medium (iCell Bioscience Inc., China). Subsequently, the cells were transferred to sterilized dAM and incubated at 37 °C for 24h before being transferred to new culture plates. Harvesting of cells was performed after 48h for subsequent analysis. Fig. 1B demonstrates that there was no significant difference in cell morphology

between dAM and control groups. Cell viability staining revealed no significant difference in apoptotic cell numbers between dAM and control groups (Figs. 1B and C). Immunofluorescent staining of CD86 confirmed comparable expression levels between both groups (Figs. 1B and D). Conversely, immunofluorescent staining of CD163 displayed significantly higher expression of CD163 in dAM group compared to control group, with statistical significance ($P < 0.05$, Figs. 1B and E). Western blot analysis of CD86 and CD163 corroborated the results obtained from immunofluorescent staining (Fig. 1F). These findings suggest that dAM promotes polarization of macrophages towards the M2 phenotype. Considering that the amniotic membrane is an integral part of the placenta and contains an abundance of cytokine resources, the level of cytokines in the dAM is higher, which confers favorable anti-inflammatory and immunomodulatory properties. Based on these observations, we hypothesize that the cytokines present in the dAM actively participate in the process of macrophage polarization towards the anti-inflammatory M2 phenotype.

To investigate the molecular mechanisms by which dAM promotes macrophage polarization towards the M2 phenotype, we conducted RNA sequencing on macrophages from the dAM and control groups (Fig. 2A). This allowed us to identify differential genes and enriched pathways in the two groups of macrophages. We employed the DESeq2 R package for differential analysis, applying filtering criteria of $\log_2\text{FoldChange} > 1$ and $P\text{-adjust} < 0.05$ to identify significantly differentially expressed genes. Consequently, we visualized the differentially expressed genes using the heatmap

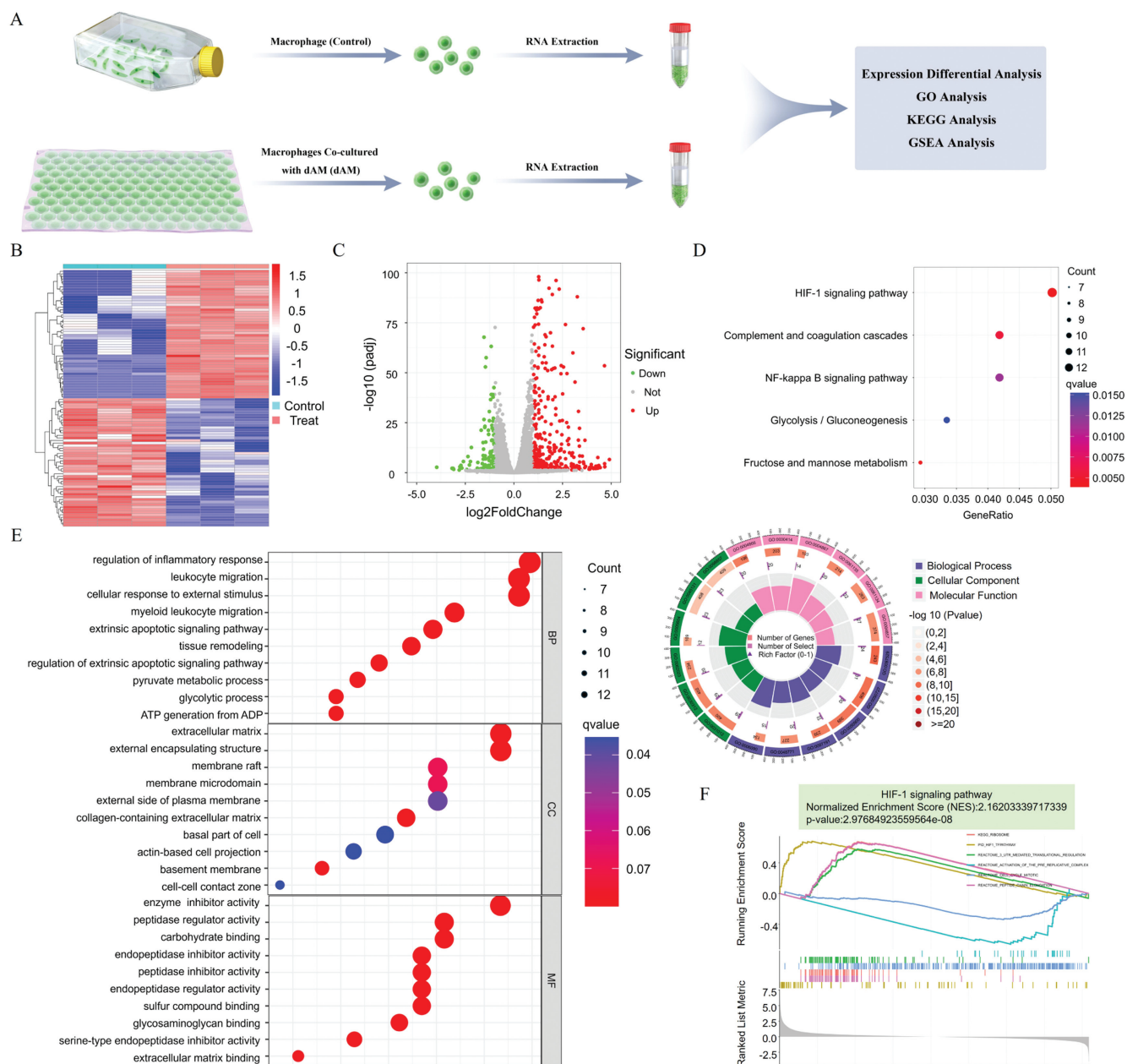


Fig. 2. Macrophages transcriptome determined by RNA sequencing. (A) Schematic overview of RNA sequencing for dAM and control groups. (B) Heat map of differentially expressed genes between dAM and control groups. (C) The volcano plot illustrating differentially regulated gene expression between dAM and control groups. (D) KEGG pathway enrichment analysis of differentially expressed genes between dAM and control groups. (E) GO classification of differentially expressed genes between dAM and control groups. (F) GSEA plots evaluating the changes of HIF-1 α signaling pathway.

R package, using red to indicate high expression and blue to indicate low expression in the respective samples (Fig. 2B). Additionally, we created a volcano plot of the differentially expressed genes using the ggplot2 R package, with red representing upregulated genes and green representing downregulated genes. Our analysis revealed 536 significantly differentially expressed genes between the two groups, of which 421 genes exhibited increased expression and 115 genes exhibited decreased expression in the dAM group compared to the control group (Fig. 2C). To gain further insights into the functional implications of the differentially expressed genes, we conducted gene ontology (GO) enrichment analysis using the clusterProfiler R package. Our filtering criteria for GO enrichment analysis were P -value < 0.05 and P -adjust < 0.05 . Notably, the differentially expressed genes showed significant enrichment in the biological processes of regulation of inflammatory

response and cellular response to external stimulus. Additionally, they were associated with various molecular functions related to enzyme activity (Fig. 2E). We also performed Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis using the clusterProfiler R package, applying filtering criteria of P -value < 0.05 and P -adjust < 0.05 . This analysis revealed significant enrichment of the differentially expressed genes in the HIF-1 α signaling pathway (Fig. 2D). Furthermore, our GSEA analysis using the RGSEA R package indicated that the HIF-1 α signaling pathway was significantly activated in the dAM group compared to control group, with statistically significant differences ($P < 0.05$) (Fig. 2F). These findings partially support our hypothesis that the cytokines abundant in the dAM, as external stimuli, participate in the regulation of the inflammatory response. Additionally, they suggest that the molecular mechanisms underlying the promotion of macrophage

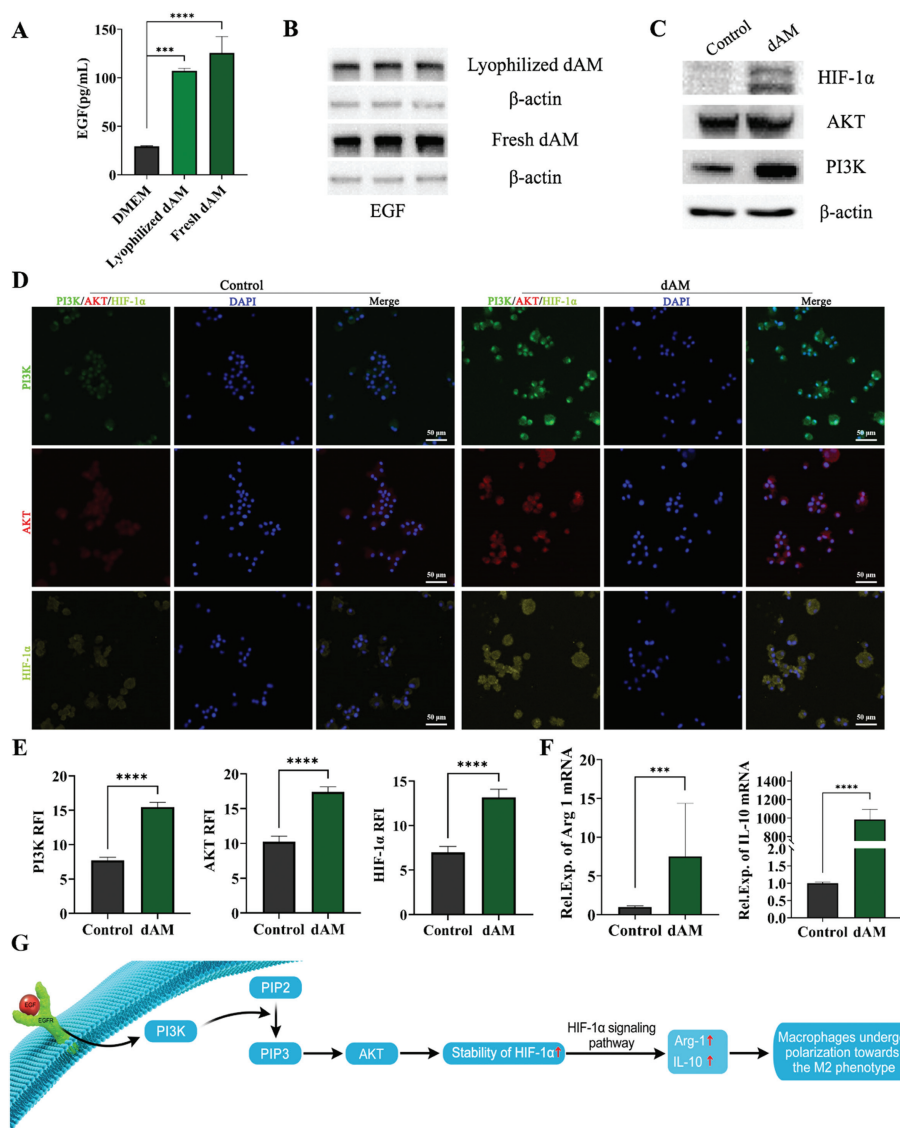


Fig. 3. dAM promotes the anti-inflammatory response of macrophages via PI3K/AKT/HIF-1 α pathway. (A) ELISA analysis of EGF in both fresh and lyophilized dAM. (B) Western blot of EGF in both fresh and lyophilized dAM. (C) Western blot of PI3K, AKT and HIF-1 α for macrophage co-cultured with dAM. (D) Representative immunofluorescence images of PI3K, AKT and HIF-1 α for macrophage co-cultured with dAM. (E) The relative fluorescence intensity (RFI) of PI3K, AKT and HIF-1 α for immunofluorescence images. (F) Relative mRNA expression of Arg-1 and IL-10. (G) The released EGF by dAM promotes the anti-inflammatory response of macrophages via PI3K/AKT/HIF-1 α pathway. Data are presented as mean \pm SD ($n=3$), one-way ANOVA. *** $P < 0.001$, **** $P < 0.0001$.

polarization towards the M2 phenotype by dAM may be associated with the cytokines present in the dAM, and this process is closely linked to the HIF-1 α signaling pathway.

HIF-1 α , a pivotal cellular protein and subunit of HIF-1, plays a crucial role in orchestrating cellular adaptive responses to varying oxygen levels. Our investigation reveals that dAM facilitates the polarization of macrophages towards the anti-inflammatory M2 phenotype through the HIF-1 α pathway. Under normoxic conditions, HIF-1 α undergoes proteasomal degradation, thereby maintaining a lower cellular presence. Based on previous studies, we postulate that dAM releases specific cell factors, augmenting the stability of HIF-1 α under normoxic conditions and inducing the macrophages' polarization towards the anti-inflammatory M2 phenotype [12–14]. To scrutinize this phenomenon, we procured extracts from both fresh and freeze-dried dAMs. Enzyme-linked immunosorbent assay (ELISA) and Western blot analyses unveiled a significantly elevated expression of epidermal growth factor (EGF) in both fresh and freeze-dried samples compared to the control group (Figs. 3A and B). Furthermore, Western blot and immunofluorescence stain-

ing demonstrated that dAM, relative to the control group, upregulates the expression of phosphatidylinositol 3-kinase (PI3K, green), protein kinase B (AKT, red), and HIF-1 α (yellow) in macrophages (Figs. 3C–E). This observation implies that the released EGF by dAM initiates the activation and autophosphorylation of epidermal growth factor receptor (EGFR), subsequently activating the PI3K/AKT pathway, culminating in the conversion of phosphatidylinositol 4,5-bisphosphate (PIP₂) to PIP₃ and the ultimate activation of AKT. Activated AKT, in turn, directly phosphorylates the HIF-1 α protein, preventing its degradation by the proteasome and eliciting additional biological effects. RT-PCR results signify a significantly higher gene expression of anti-inflammatory factors Arg-1 and IL-10 in macrophages from the dAM group compared to the control group (Fig. 3F). Consequently, the released EGF by dAM stabilizes HIF-1 α under normoxic conditions through the PI3K/AKT pathway, activating the HIF-1 α pathway, upregulating the expression of anti-inflammatory factors Arg-1 and IL-10 in macrophages, and inducing the polarization of macrophages towards the anti-inflammatory M2 phenotype (Fig. 3G).

Nowadays, dAM, a natural biological material, has garnered significant attention for its role in fostering tissue repair and modulating the inflammatory process. Our research suggests that EGF released from dAM plays a pivotal role in these processes. Serving as a growth factor, EGF can initiate the PI3K/AKT pathway through binding to cell surface receptors, thereby influencing cell survival, proliferation, and metabolism. Activation of this signaling pathway has been observed to enhance the stability of HIF-1 α , subsequently activating the HIF-1 α pathway. Through augmenting the stability of HIF-1 α , dAM can prompt macrophages to polarize towards M2 macrophages. M2 macrophages are commonly associated with anti-inflammatory and reparative processes, and the heightened expression of the anti-inflammatory factors Arg-1 and IL-10 further substantiates their capacity to regulate immune responses. This discovery bears significant clinical implications, particularly in the domain of promoting tissue repair. Nevertheless, further research is imperative to delve into the intricate dynamic regulatory mechanisms and effects under diverse pathological conditions, ensuring both safety and effectiveness. In summary, this study furnishes a crucial theoretical foundation for a more profound comprehension of the role of dAM in immune regulation and tissue repair.

This study investigates the molecular mechanisms through which dAM exerts anti-inflammatory effects during the tissue repair process. Specifically, it elucidates the roles of EGF release, PI3K/AKT pathway activation, enhanced stability of HIF-1 α , and HIF-1 α pathway activation in promoting macrophage polarization towards the M2 phenotype and exerting anti-inflammatory effects. Overall, our research enhances the understanding of the immunomodulatory function of dAM in tissue repair, offering substantial theoretical underpinning for its potential therapeutic applications in promoting tissue regeneration. Nonetheless, future investigations should further elucidate its molecular mechanisms, refine treatment strategies, and validate its feasibility in more intricate *in vivo* settings.

Declaration of competing interest

The authors declare the following competing financial interest(s): Dr. Juan Wu (a co-author) is an employee of Wuhan

Kangchuang Biotechnology Limited that funded this study. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit authorship contribution statement

Xiongbo Song: Data curation, Formal analysis. **Jinwen Xiao:** Data curation. **Juan Wu:** Data curation, Funding acquisition. **Li Sun:** Conceptualization, Data curation. **Long Chen:** Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

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