Received: 23 March 2023 | Revised: 2 June 2023 | Accepted: 29 June 2023

DOI: 10.1111/jocd.15928

JCD Journal of Cosmetic Dermatology WILEY

ORIGINAL ARTICLE

Biostimulating fillers and induction of inflammatory pathways: A preclinical investigation of macrophage response to calcium hydroxylapatite and poly-L lactic acid

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Abstract

Introduction: Initial macrophage response to biostimulatory substances is key in determining the subsequent behavior of fibroblasts and the organization of newly synthesized collagen. Though histological studies suggest that calcium hydroxylapatite (CaHA) filler initiates a regenerative healing response with collagen and elastin deposition similar to natural, healthy tissue rather than an inflammatory response with fibrosis, the relative activity of macrophages stimulated by CaHA, as well as how this activity compares to that induced by other biostimulatory fillers, has not been explored. The aim of the study is to characterize the in vitro macrophage response to two biostimulory fillers, CaHA and PLLA (poly-L lactic acid), and to evaluate their inflammatory potential.

Methods: Primary human macrophages were incubated with two dilutions (1:50 and 1:100) of commercially available CaHA or PLLA. After 24 h incubation, an inflammation array was used to screen for the expression of 40 cytokines, released by macrophages. ELISA was used to confirm array results.

Results: Four cytokines were significantly upregulated in M1 macrophages incubated with PLLA compared to both unstimulated controls and CaHA: CCL1 ($p < 0.001$), TNFRII ($p < 0.01$), MIP-1α ($p < 0.05$), and IL-8 ($p < 0.001$). In M2 macrophages, MIP-1α ($p < 0.01$) and MIP-1β ($p < 0.01$) were significantly upregulated by PLLA compared to CaHA and unstimulated controls.

Conclusion: Together, these findings indicate that the CaHA mode of action is a non-inflammatory response while PLLA initiates expression of several cytokines known to play a role in inflammation. Our study supports the concept that these two "biostimulatory" fillers follow distinct pathways and should be considered individually with regard to mechanism of action.

KEYWORDS
CaHA, calcium hydroxylapatite, dermal filler, poly-L-lactic acid, regenerative

1 | INTRODUCTION

Calcium hydroxylapatite (CaHA, Radiesse®; Merz North America, Raleigh, NC) is a biocompatible, biodegradable, and resorbable biostimulatory filler consisting of calcium hydroxylapatite (CaHA) microspheres (25–45 μm diameter) suspended in a 70% aqueous carboxymethylcellulose gel carrier. Approved indications include moderate-to-severe facial wrinkles, such as nasolabial folds and marionette lines; enhancing fullness of the cheeks and other facial contours; improving volume in hands; and improvement of jawline contour.[1–4] However, CaHA is often used off-label for revolumization of other areas of the face and body and is also used in a diluted form for skin tightening.[1,3,5] While physiochemically distinct,[6] in the clinic CaHA is often considered alongside poly-L-lactic acid (PLLA; Sculptra®, [Galderma, Lausanne, Switzerland]) as an option for biostimulation-based revolumization.

CaHA and PLLA differ in several regards. First, the two fillers are mechanistically distinct. CaHA filler provides immediate volume replacement via carrier carboxymethylcellulose gel, which is subsequently replaced over time by native collagen induced by the CaHA microspheres.[7] This neocollagenesis maintains the volume provided initially by the carrier gel, and also improves skin quality and thickness.[8,9] In contrast, PLLA creates volume exclusively through promotion of a subclinical foreign body inflammatory response, which promotes collagen synthesis that becomes apparent as volume over the course of several months.[10] For PLLA, volume increases seen immediately after injection are related to swelling and the suspension of microparticles and wane within a few hours to a few days.[6]

In addition, CaHA and PLLA are chemically distinct and lead to neocollagenesis through different pathways. Immune pathways are determined early following injection and involve differential recruitment of immune cells. Immediately after injection, material is surrounded by blood from injured vessels, lipids, sugars, ions, and proteins (e.g., albumin, fibrinogen, fibronectin, vitronectin, and gammaglobulins), which are adsorbed on the particle surface within minutes. Even at this very early stage, the different surface properties of CaHA and PLLA likely lead to attachment of distinctive collections of extracellular proteins and a divergence of pathways leading to neocollagenesis.[11] CaHA particles function as a scaffold to support resident fibroblast function and induce pathways for multiple aspects of extracellular matrix (ECM) regeneration such as HA synthesis, angiogenesis, and organization of collagen, elastin, and proteoglycans.[12] Additionally, histologic studies suggest minimal infiltration of inflammatory cells, potentially leading to restoration of normal skin structure and function.[13–15]

The PLLA tissue response appears to be more inflammatory in nature, and protein adsorption is followed by neutrophil and macrophage infiltration from Day 2 to Day 10 after injection. By 1 month, mast cells, mononuclear macrophages, foreign body cells, and lymphocytes surround PLLA microparticles, and as a result of this foreign body reaction, PLLA particles are encapsulated by more immature, less organized collagen type III while more organized, and mature collagen type I is not found in close proximity to the PLLA particles or surrounding cellular infiltrate and is observed only at the periphery of the granulomatous reactions.[6,11,16] These differential immune cell responses and distinct patterns of neocollagenesis suggest distinct modes of action that likely occur through early divergence of tissue response pathways.

Macrophage activity dictates the behavior of fibroblasts and is critical for the recruitment of immune cells and the signaling cascades that support fibroblast migration and activity, proliferation, differentiation, and/or profibrotic pathways.[17] In this study, the activity of M1 and M2 macrophages and the cytokine expression profile induced by CaHA and PLLA was examined in efforts to better understand macrophage response to these materials.

2 | METHODS

2.1 | Fillers and controls

CaHA (Radiesse®, Merz North America, Raleigh, NC, USA) and PLLA (Sculptra®, Galderma, Lausanne, Switzerland) were purchased and tested in 96-well culture microplate human microphage assays. Lipopolysaccharide (LPS) 100 ng/mL from *Escherichia coli* and human recombinant interleukin-4 (IL-4) 20 ng/mL were included as controls for the M1 and M2 macrophage populations, respectively. M1-Macrophage Generation Medium DXF was used to culture M1 macrophages, and M2-Macrophage Generation Medium DXF was used to culture M2 macrophages (Promocell, Heidelberg, Germany).

2.2 | Cell culture

M1 (hMDM-GMCSF(-)-c single donor M1 macrophages [Promocell, Heidelberg, Germany]) and M2 (hMDM-GMCSF(-)-c single donor M2 macrophages [Promocell, Heidelberg, Germany]) were thawed and allowed to recover for 20 min in cold medium. Cells were centrifuged at 350×*g* for 15 min at room temperature, and the cell pellet was resuspended into the appropriate medium. Cell counts were adjusted to 100000 cells/cm^2 for M1 macrophages and 200000 cells/cm^2 for M2 macrophages. Cells were incubated overnight in a 96-well plate at 37°C. The cell culture medium was changed and reduced from 150 μL/well to 50 μL/well. At 24 h after initial plating, 100 μL of medium containing filler diluted with medium (1:50 and 1:100 Radiesse; 1:50 and 1:100 Sculptra) or corresponding volumes of medium alone were transferred into assay wells to reach a final assay volume of 150 μL/well.

2.3 | Supernatant analysis

Cells were exposed to filler for 24 h. Supernatants were pooled and volume normalized to account for inter-well evaporation effects and then frozen at −80°C until analysis. Samples were centrifuged at 10000×*g* for 10 min and screened for 40 human cytokines using the RayBio® Human Inflammation Array GS3 (RayBiotech, Peachtree Corners, GA) according to the manufacturer's protocol. Due to the minimal capacity for these filler products to initiate significant cytokine release

compared to established inflammatory substances, the levels of cytokine release observed were low. For this reason, in sample results where a trend was observed from the screening array, quantitative ELISA was used to confirm the presence or absence of significance (IL-8 [Abcam ab214030], MIP-1α [Abcam ab214569], and MIP-1β [Abcam ab100597]). Statistical analysis was performed using one-way ANOVA with the Turkey's multiple comparison test, with single pooled variance. All experiments were conducted at least in triplicate.

3 | RESULTS

3.1 | M1 macrophages

None of the 40 tested cytokines in the screening showed increased expression in M1 macrophages after incubation with CaHA compared with the unstimulated controls. However, cultures from M1 macrophages incubated with PLLA demonstrated significantly higher levels of four cytokines compared with the unstimulated control and CaHA dilutions (Figure 1): chemokine (C-C motif) ligand 1 (CCL1), soluble tumor necrosis factor receptor II (sTNFR2), and macrophage inflammatory protein alpha (MIP-1α) (Figure 1). Significance for MIP-1α was confirmed via ELISA, and trends for interleukin 8 (IL-8) observed in the screening assay were evaluated with ELISA and shown to be significant.

Mean levels of CCL1 in the M1 macrophages after incubation with PLLA dilutions of 1:50 (31 880 mean fluorescence units [MFU]) and 1:100 (27 550 MFU) were significantly higher than levels detected after incubation with CaHA dilutions of 1:50 (10 255 MFU) and 1:100 (8373 MFU) ($p < 0.001$ and $p < 0.001$, respectively; Figure 1). TNFRII activity, indirectly measured via levels of sTNFR2, was significantly higher after incubation with PLLA dilution of 1:50 (12 832 MFU) than after incubation with CaHA dilutions of 1:50 (4877 MFU) and 1:100 (4008 MFU) ($p < 0.01$ and $p < 0.01$, respectively). MIP-1α levels

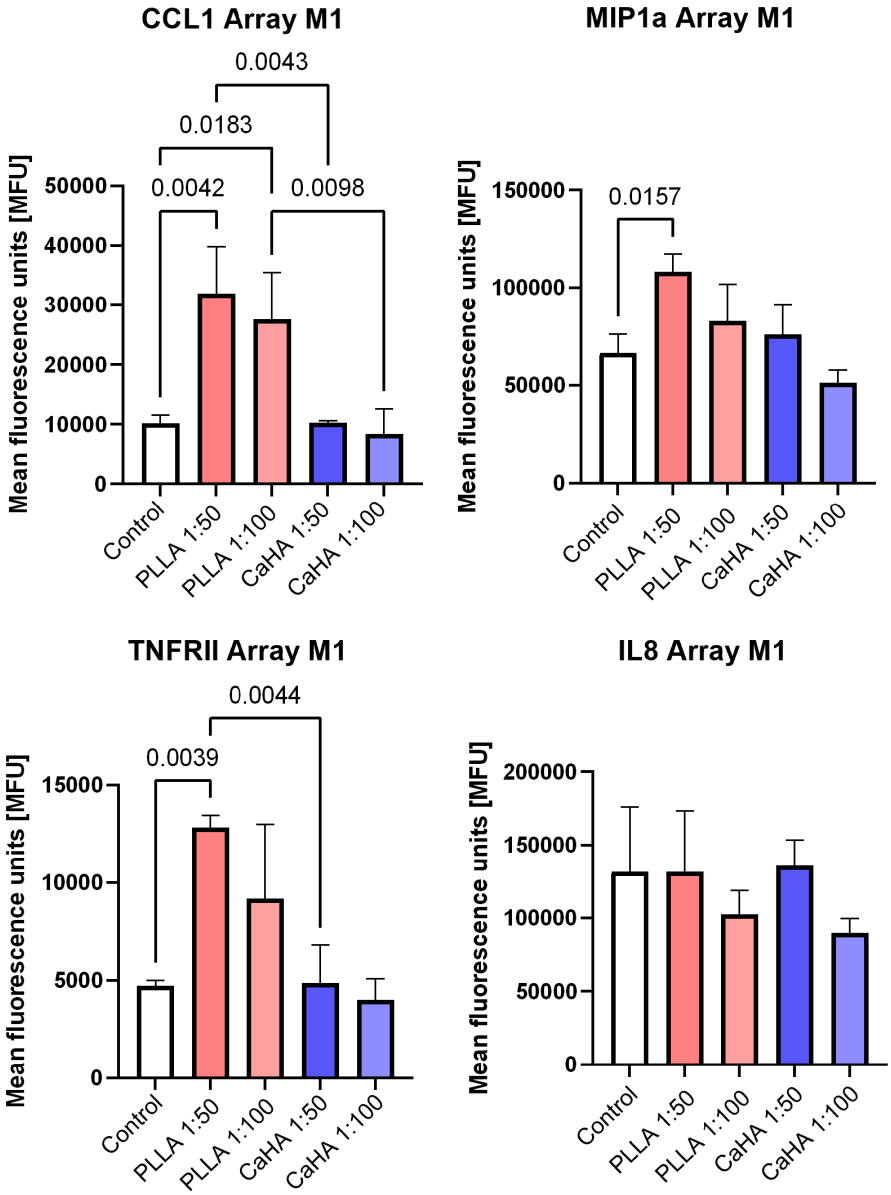

FIGURE 1 Cytokine levels in M1 macrophages after 24 h incubation with CaHA or PLLA identified with the human inflammation array.

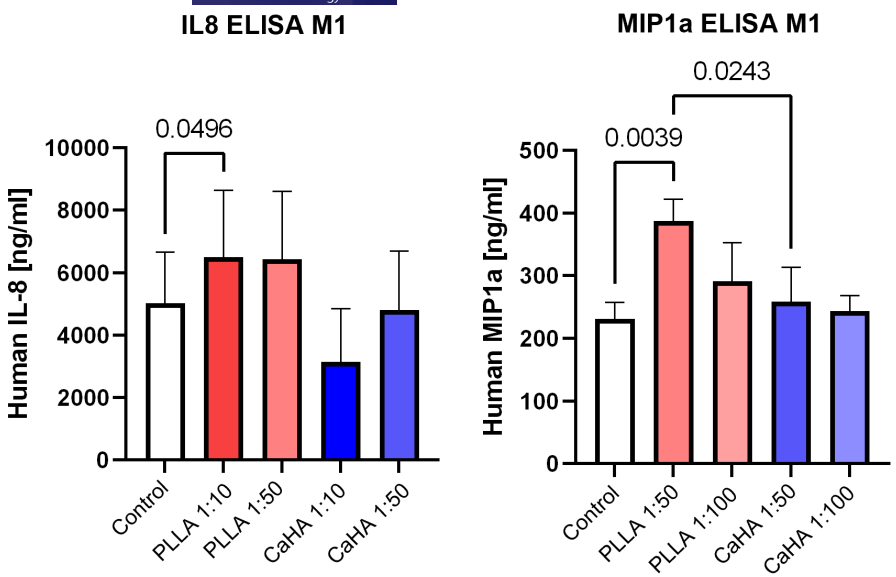

FIGURE 2 Cytokine levels in M1 macrophages after 24 h incubation with CaHA or PLLA confirmed with ELISA.

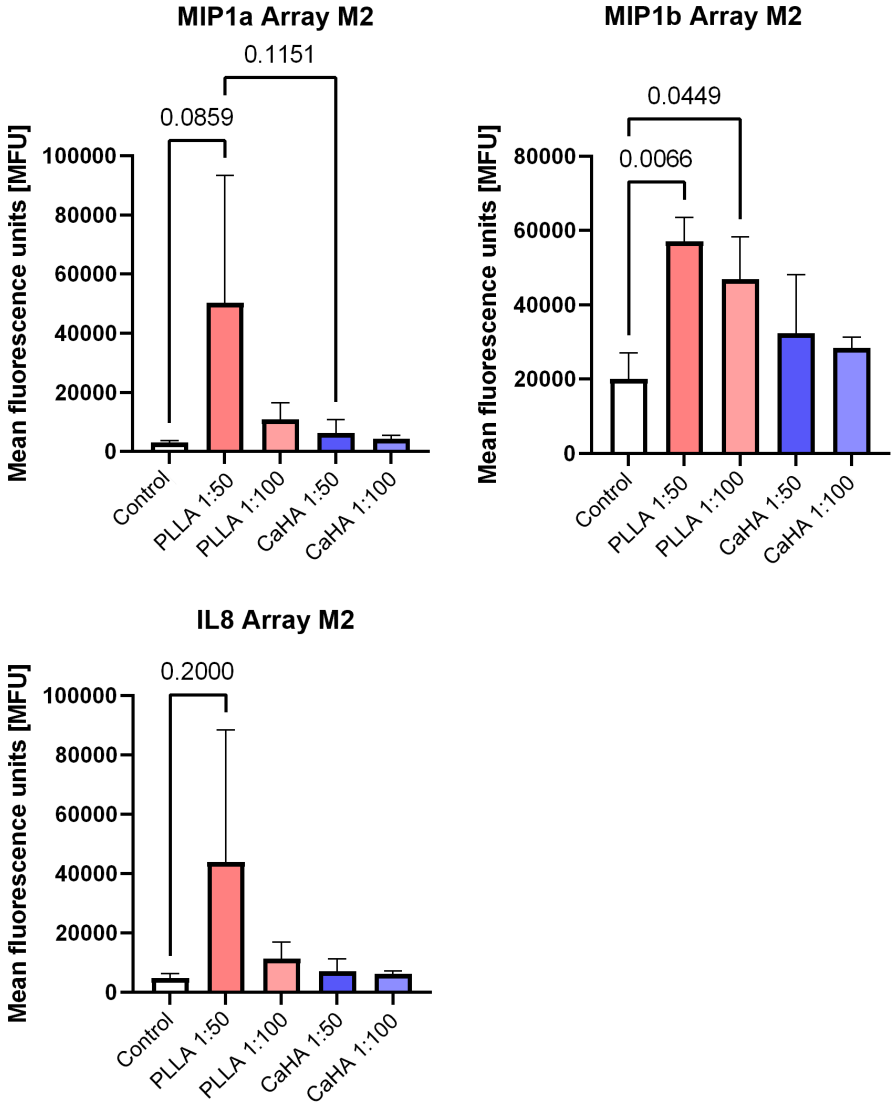

FIGURE 3 MIP-1α and MIP-1β cytokine levels in M2 macrophages after 24 h incubation with CaHA or PLLA identified with the human inflammation array.

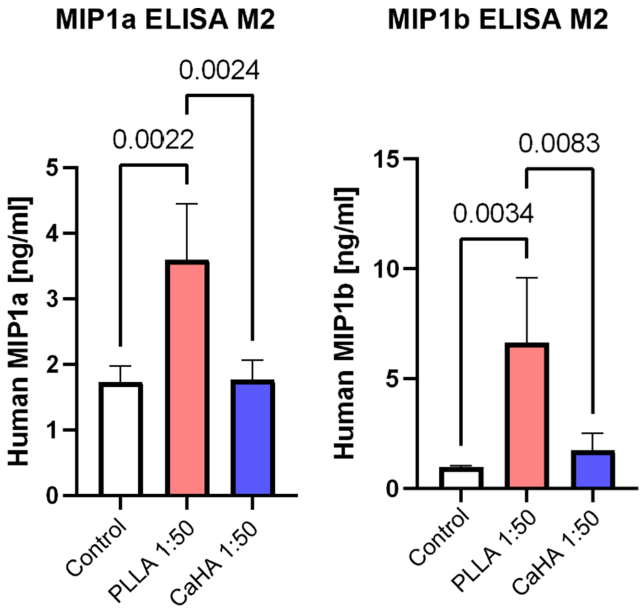

FIGURE 4 Cytokine levels in M2 macrophages after 24 h incubation with CaHA or PLLA confirmed with ELISA.

after incubation with PLLA 1:50 dilution (50 172 MFU) were significantly higher than the control. This was confirmed via ELISA, which showed a significant difference in expression between CaHA 1:50 and PLLA 1:50 (258.7 ng/mL and 387.7 ng/mL) (Figure 2). IL-8 levels after incubation with PLLA dilution of 1:50 (6430 ng/mL) were also significantly higher than levels detected after incubation with CaHA dilutions of 1:50 (4805 ng/mL) and 1:10 (3149 ng/mL; $p < 0.001$ and $p < 0.001$, respectively).

3.2 | M2 macrophages

Similar to the results with the M1 macrophages, none of the 40 cytokines in the screening showed increased expression after incubation of M2 macrophages with CaHA compared with the unstimulated control. However, the MIP-1β showed significantly elevated expression after incubation of M2 macrophages with PLLA (Figure 3). Elevated levels for MIP-1β and MIP-1α were confirmed by ELISA (Figure 4). IL-8 was elevated for PLLA 1:50 compared to CaHA 1:50 and controls, but this did not reach statistical significance in the screening assay.

Mean levels of MIP-1α after incubation with PLLA dilution of 1:50 (3.6 ng/mL) were significantly higher than after incubation with CaHA dilution of 1:50 (1.7 ng/mL). MIP-1β levels after incubation with PLLA dilution of 1:50 (6.648 ng/mL) were significantly higher than after incubation with CaHA dilution of 1:50 (1.746 ng/mL).

4 | DISCUSSION

Cytokines and chemokines comprise a large group of proteins that coordinate the immune response throughout the body. M1 macrophages can phagocytose dead cells and bacteria, but also secrete proteins that promote the deposition of collagen, angiogenesis, the formation of granulation tissue, re-epithelization, and the activation of other immune cells. Previous studies have found that in vitro stimulation with IFN-γ and LPS of M1 macrophages induces a pro-inflammatory cytokine production profile, including TNF-α, IL-1, and IL-6.[18,19] In contrast, M2 macrophages respond by producing lower levels of pro-inflammatory cytokines such as IL-12, IL-18, and TNF-α and higher levels of anti-inflammatory cytokines TGF-β and IL-10, which suggests a more regenerative role in the healing process.[18]

In this preclinical study, none of the 40 cytokines tested showed significantly increased expression in M1 or M2 macrophages when incubated with CaHA beyond that exhibited by the negative control. In contrast, when stimulated by PLLA, 4 cytokines showed significant elevations in M1 macrophages and 2 in M2 macrophages. These results suggest a low inflammatory potential for CaHA compared to PLLA, which is consistent with previous research that failed to identify an inflammatory response to the CaHA gel matrix.[14]

In this study, the cytokines found to increase in cell culture with M1 or M2 macrophages supplemented with PLLA, but *not* CaHA were sTNFR2, CCL1, MIP-1α, MIP-1β, and IL-8. Each of these cytokines has a direct role in inflammation. sTNFR2 is cleaved upon activation of TNFRII and thus acts as a proxy for measurement of receptor activity.[20] TNFRII is one of the receptors for tumor necrosis factor α (TNFα), an essential signaling protein in the innate and adaptive immune system that plays a critical role in the upregulation and downregulation of regulatory T-cell (Treg) activity. Evidence suggests that TNFRII contributes to immune modulation through cell activation and the recruitment and proliferation of immune cells.[21–27] CCL1 is a chemokine produced by activated monocytes/macrophages, T lymphocytes, and endothelial cells that mainly is a chemoattractant for monocytes/macrophages, lymphocytes, and neutrophils.[28–32] It is thought to play a major role in inflammatory processes,[33] and given the results of the experiments in this study, PLLA may induce CCL1 expression by macrophage activation. MIP-1α (also known as CCL3) is an inflammatory, chemotactic chemokine known to be secreted by macrophages that recruits inflammatory cells, promotes wound healing, and maintains the effector immune response. Cells that release MIP-1α are increased at sites of inflammation and help to recruit macrophages, lymphocytes, and eosinophils.[34,35] Like MIP-1α, MIP-1β (also known as CCL4) is a chemotactic chemokine that promotes inflammation through the use of a shared receptor, CCR5. CCR5 is expressed on macrophages, dendritic cells, and activated T helper 1 cells, resulting in the recruitment of monocytes/macrophages, natural killer cells, and T-cell populations.[34,36–38] IL-8 is widely used as a diagnostic marker for traumatic, inflammatory conditions and is known to contribute to tumor progression.[39–41] IL-8 is known to contribute to the induction of chemotaxis by neutrophils, lymphocytes, monocytes, and macrophages.[42,43]

Taken together, these markers suggest a heightened inflammatory profile for PLLA as well as a consistent absence of inflammatory activity for CaHA. This differential inflammatory response supports our current understanding of CaHA's mechanism of

action following pathways that are different to those of PLLA, providing an environment that is conducive to regeneration of the organized architectural elements of the extracellular matrix rather than the encapsulation and fibroplasia type deposition of collagen that is seen in the foreign body response to PLLA.[6,15,44] There are various working hypotheses to explain the apparent unique mode of action of CaHA that diverge from the foreign body response of PLLA and other biostimulatory formulations. Among these hypotheses are published models of CaHA acting as a calcium sink utilizing chemoattraction to upregulate fibroblasts,[45,46] and neocollagenesis via direct interactions between fibroblasts and CaHA microspheres.[47,48] Our knowledge today indicates that there are complex immunological pathways and interactions at play in all biostimulating formulations that are unique to each, as supported by this in vitro study. The various pathways are likely to result in differing outcomes changing, for example, the ratios of collagen types and other elements of the extracellular matrix, such as elastin and glucosaminoglycans, which will ultimately influence skin and soft tissue structure and function. Immunological pathways and outcomes will, of course, also have some variation in vivo depending on the genetic, aging, and possible pathological milieu in which they occur.

The present study revealed a differential response to these fillers by both M1 and M2 macrophages and offers evidence that an inflammatory foreign body reaction is largely absent for CaHA, while there are several inflammatory markers expressed by PLLA-stimulated macrophages. Differentiating fillers on the basis of immune cell activity and subsequent fibroblast activity may have important clinical implications. Especially as uses for biostimulatory fillers expand to include treatment of a wider area for skin tightening and is used in multiple areas of the body, it is important to understand the nature of the collagen deposition induced by available treatments and to explore the impact of these differences on clinical outcomes in future studies. This fascinating area of research requires further study to elucidate these pathways and provide the data that will enable clinicians to select appropriate interventions tailored to their individual patients.

5 | CONCLUSION

Cytokine levels in human M1 and M2 macrophages after incubation with CaHA were similar to controls, indicating that CaHA has a non-inflammatory potential. Together with previous histological studies that indicate CaHA does not initiate an inflammatory response, this study provides further evidence to support the hypothesis that CaHA's mode of action follows a more regenerative pathway, and PLLA follows a more inflammatory foreign body response.

AUTHOR CONTRIBUTIONS

Study conception and design: Bartosch Nowag, Thomas Hengl, and Daniela Schäfer; data collection: Bartosch Nowag and Daniela Schäfer; analysis and interpretation of results: Bartosch Nowag, Thomas Hengl, Daniela Schäfer, Niamh Corduff, and Kate Goldie; manuscript draft review/editing: Bartosch Nowag, Thomas Hengl, Daniela Schäfer, Niamh Corduff, and Kate Goldie. All authors reviewed the results and approved the final version of the manuscript.

ACKNOWLEDGMENTS

Medical writing assistance was provided by Ginny Vachon, PhD, and Cecelia Wall of Principal Medvantage, LLC, Atlanta, Georgia under the direction of the authors. Funding for this support was provided by Merz Aesthetics.

FUNDING INFORMATION

Funding for this research was provided by Merz Aesthetics.

CONFLICT OF INTEREST STATEMENT

This study was funded by Merz Aesthetics, GmbH, Frankfurt am Main, Germany. Dr. B. Nowag, Dr. T. Hengl, and D. Schaefer are employees of Merz Aesthetics. Dr. N. Corduff and Dr. K. Goldie have been consultants and/or speakers for Merz Aesthetics. The authors report no other potential conflicts of interest for this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

No human or animal subjects were used in this study.

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How to cite this article: Nowag B, Schäfer D, Hengl T, Corduff N, Goldie K. Biostimulating fillers and induction of inflammatory pathways: A preclinical investigation of macrophage response to calcium hydroxylapatite and poly-L lactic acid. *J Cosmet Dermatol*. 2023;00:1-8. doi:10.1111/jocd.15928