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ORIGINAL CONTRIBUTION

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Dermal fillers do not induce upregulation of NLRP3 inflammasomes or expression of inflammatory cytokines in granulomas

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Abstract

Background: Filling materials have increasingly been used in aesthetics over the last decades. Understanding the pathophysiology of granuloma formation as a very relevant unwanted side effect of filler application may be essential to help avoid these adverse events.

Aims: Our aim was to investigate the role of the inflammasome in the formation of filler granuloma, as a central column of the innate immune response.

Methods: RPMI 1640 medium was used for growth of THP-1 cells and the induction of THP-1 macrophages. Sonication was applied in order to crush the acrylic particles of the filler. ELISA was the method of analysis for the specific cytokines. Biopsy specimens of filler granuloma were analyzed by various immunohistochemical methods. GraphPad Prism 5 software was used for the statistical data analysis.

Results: Neither was the sensor NALP3 overexpressed, nor could an elevated expression of cleaved IL-1β, IL-18, or IFN-γ be detected. Furthermore, no increased expression of IL-8 or IL-1β was detectable in vitro.

Conclusion: No increased inflammasome activation could be observed; however, filler granulomas were infiltrated with granulocytes and macrophages. Therefore, we speculate that an unspecific immune response might be the key player in the formation of filler granuloma.

KEYWORDS

caspase, fillers, granuloma, inflammasome, interleukins

1 | INTRODUCTION

During the last decades, the importance of having a firm, juvenile facial skin without wrinkles has increased significantly. A plethora of different approaches to soften wrinkles and augment soft tissue has been established in aesthetic medicine over the last years. Filler materials are still the gold standard to recreate volume in an aging face (eg, rebuilding cheeks) and are commonly applied for the elimination

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of deeper wrinkles. Various types of materials have been used to create long-lasting results.[1,2] Moreover, the augmentation of the facial soft tissue and rejuvenation procedures using different filler materials are widely performed for cosmetic enhancement because of their highly predictable and pleasing outcomes.[3,4] Additionally, the number of indications apart from facial applications has exponentially increased.[2]

Despite of the benefits of successful filler treatments, the number of associated complications is likely to grow during the next few years. Unfortunately, many injectable-applying physicians still lack in-depth knowledge of the anatomical background as well as product and technique related complications associated with minimally invasive procedures.[1]

Even though the manufacturers of permanent injectable fillers have always claimed that their products are widely inert, biocompatible, nontoxic, and nonimmunogenic, there are some materials ranging from polymer hydrogels without microparticles to combination products that use a collagen suspension or a hyaluronic acid gel as a basis with incorporated polymer microspheres or polygonal particles.[2] The filling effect of polymer gels is based on the volume injected. Regarding the combination gels, it depends partly on the volume injected and partly on the intended host. In the past, the patients' desire for long-lasting results has led to an increased use of these combination products, such as Dermalive® (Dermatech), which is one of the filling materials with the most frequent adverse foreign body reactions which patients unfortunately still suffer from, although it was taken off the European market in 2007. It consists of hyaluronic acid gel with polygonal acrylate particles.[5,6] Those filler materials containing 2-hydroxyethyl methacrylate and ethyl methacrylate monomer units embedded in hyaluronic acid are known for their strong potential to induce granulomas.[5,7] Therefore, we chose this particular product to be the representative example of filling materials in our investigation of the immune response in the formation of the filler induced granulomas.[8,9]

The inflammasome is an intracellular protein complex, which is important for the detection of danger-associated molecular patterns (DAMPs), like bacteria and crystalloid organic or inorganic structures. As cells from the mononuclear phagocyte system (MPS) are involved in the formation of granuloma, it might also be the case in the filler granuloma, where the inflammasome could play an important role, too.[10,11]

2 | MATERIALS AND METHODS

2.1 | Cell culture and induction of THP-1 macrophages

THP-1 cells (human monocytic cell line with the ability to differentiate to macrophage-like cells upon induction) were grown in RPMI 1640 (leukocyte growth medium, developed at the Roswell Park Memorial Institute) supplemented with 20 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and L-glutamine (Sigma) containing 10%-20% fetal calf serum (FCS; Sigma) and 1% PSN (~5000 units penicillin, 5 mg streptomycin and 10 mg neomycin/mL; Sigma) at 37°C with 5% CO_2. To induce monocyte-to-macrophage differentiation (THP-1 macrophages), the THP-1 cells (3×10^5 cells/well) were cultured on 24-well plates (1 mL/well) for 72 hours in the standard culture medium (10% FCS RPMI + PSN) supplemented with 10, 50, and 100 nmol/L (6.25, 31.3, and 62.5 ng/mL) phorbol 12-myristate 13-acetate (PMA; Sigma).[12]

2.2 | Filler

Dermalive® (Dermatech) is a cosmetic facial filler made of hyaluronic acid with acrylic hydrogel particles. It is considered a semi-permanent facial filler, lasting several years as opposed to several months. After becoming evident that this biphasic filler would cause late granulomas at numerous cases, its license as a medical product had to be suspended.[7,13,14]

2.3 | Sonication

In order to break the acrylic hydrogel particles of the filler, sonication was performed with an immersion bath sonicator Sonorex RK100 device (BANDELIN Electronic GmbH & Co. KG) for a total of 60 minutes (six cycles of 10 minutes, followed by 5 minutes cooldown periods, 230 V, 0.4 A, 35 kHz) or with a direct contact probe sonicator Sonifier 250 (Branson Ultrasonics BV) for a total of 45 minutes (three cycles of 10 minutes and 1 cycle of 15 minutes, followed by 10 minutes rest periods, duty cycle 20%, output control 10).

2.4 | Enzyme-linked immunosorbent assay (ELISA)

The PMA-induced THP-1 macrophages were subjected to the filler in different concentrations ranging from 1:10 000 to 1:100 for 24-72 hours at 37°C with 5% CO_2. Supernatants were then analyzed by enzyme-linked immunosorbent assay (ELISA) for IL-1β (IL-1β ELISA Duo Set; R&D Systems Inc) and IL-8 ELISA (R&D Systems).

2.5 | Patient material

For diagnostic purposes, indicated biopsies of filler granulomas caused by Dermalive® were provided by L. G. Wiest and W. Stolz. A total of seven biopsies were examined. For all procedures informed written consent was obtained. All retrospective sample examinations were approved (17-666 UE) by the committees on investigations involving human subjects at the Faculty of Medicine, Ludwig Maximilian University (Munich, Germany). All histological examinations were performed by a board-certified dermatohistopathologist.

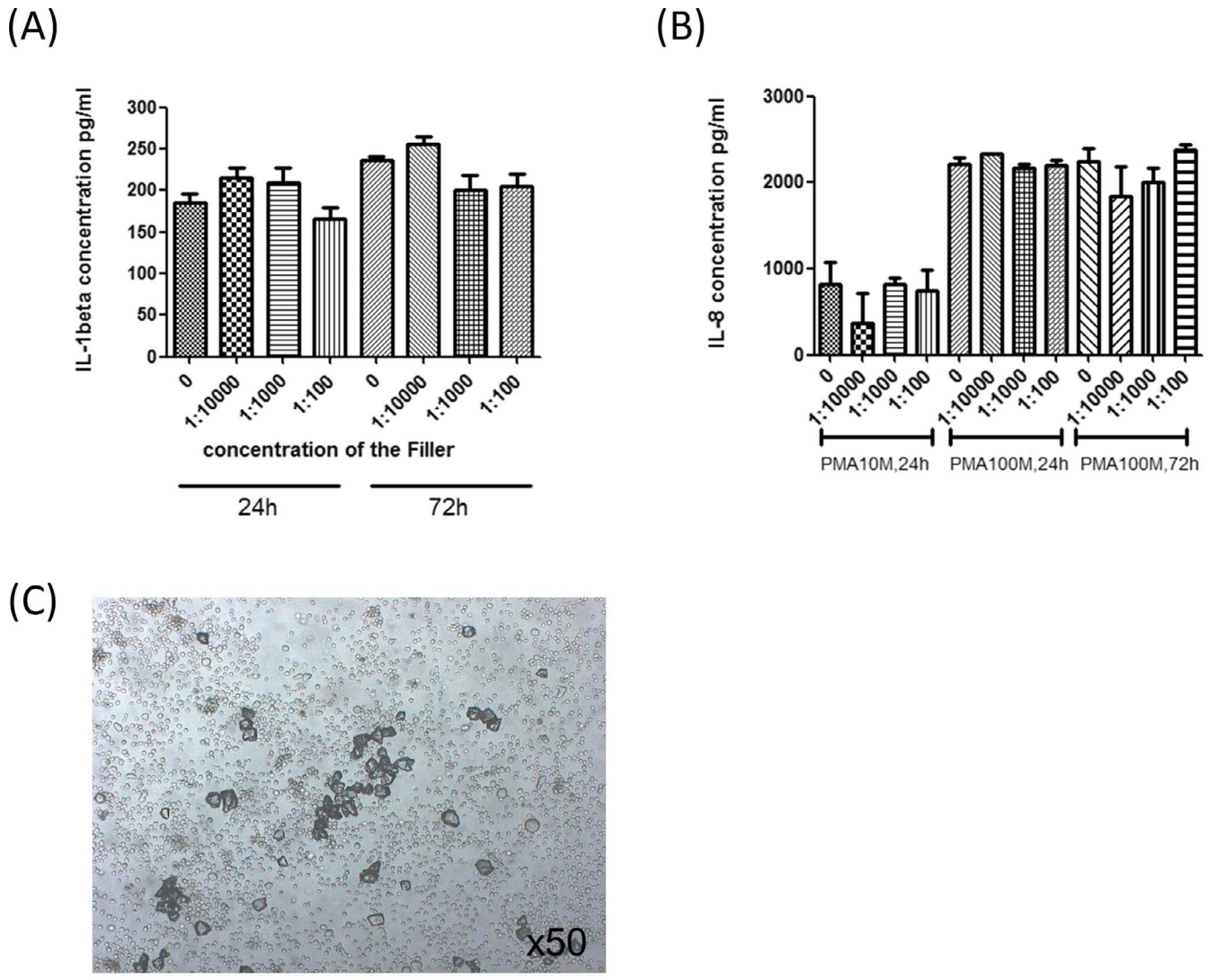

FIGURE 1 IL-1β ELISA with filler with longer incubation time THP-1 macrophages (human monocytic cell line differentiated to macrophage-like cells), which are induced by 100 nmol/L phorbol 12-myristate 13-acetate (PMA), produced under filler stimulation. There was no significant difference between longer and shorter incubation time, as well as with and without filler (A). There was no difference of IL-8 production by addition of filler (B). 72 h after stimulation of THP-1 macrophage, which is induced by 10 nmol/L PMA with 1:1000 filler, THP-1 cells seemed to proliferate again. The filler does not seem to interact with the macrophages. No phagocytosis could be observed (C)

2.6 | Immunohistochemistry

Sections of filler granuloma biopsies were incubated overnight with polyclonal cleaved IL-1β antibody (Cell Signaling Technology Europe BV). Following incubation with monoclonal mouse anti-rabbit antibody (Dako) for 30 minutes, the sections were visualized by adding Dako RealTM Detection System K5000, APAAP, Mouse (Dako). Cells were counterstained with hematoxylin, and the sections were analyzed with a TissueFAXS System microscope (Carl Zeiss AG) and TissueQuest software (TissueGnostics GmbH). Polyclonal IFN-γ antibody (Abcam) was applied using the same protocol. Stains with polyclonal IL-1β antibody (Santa Cruz Biotechnology Inc), polyclonal cleaved caspase-1 antibody (Santa Cruz), monoclonal NALP3 (NACHT, LRR, and PYD domains-containing protein 3) antibody (Abcam), and monoclonal NALP3 antibody (Enzo Life Sciences GmbH) were visualized by adding DAB (3,3'-diaminobenzidine) reagent (Dako) after incubation with a horseradish peroxidase (HRP) conjugated second antibody (Dako) for 1 hour. The used isotype controls were rabbit polyclonal IgG (Abcam), normal goat IgG (Santa Cruz), and mouse monoclonal IgG (Dako).

2.7 | Statistical analysis

The results were displayed as means ± SD. Statistical significance of the results was calculated using the Student's t test at the * equates $P = .05$, ** equates $P = .005$, and *** equates $P = .001$ significance level. All tests were performed using the GraphPad Prism 5 software (GraphPad Software Inc). A P-value of <.05 was regarded as significant.

3 | RESULTS

In cell culture, there was no difference in the expression of IL-1β, measured by ELISA, in THP-1 macrophages, which were induced by 100 nmol/L PMA compared with negative control (Figure 1). Furthermore, there was no significant difference between longer and shorter incubation times, as well as with and without filler (Figure 1A, S1). There was no difference of IL-8 production by addition of filler 72 hours after stimulation of THP-1 macrophage, which was induced by 10 nmol/L PMA with 1:1000 filler (Figure 1B). THP-1 cells seemed to proliferate again. The filler does not seem to interfere with the macrophages. No phagocytosis of filler particles could be observed, in contrast to the histological specimens described below.

Additionally, we could show that different concentrations of the hyaluronic acid/acrylic hydrogel filler did not lead to an increased IL-1β release of THP-1 macrophages (Figure 1A). Interestingly, the release of IL-8 is dependent on the phorbol myristate acetate (PMA), but not on the filler concentration (Figure 1B).

To further investigate whether the size of the microparticles was relevant, we sonified the filler in order to fragment the polyacrylate particles. We could show the filler particles before (Figure 2A) and after sonification (10 minutes for six cycles, in between 5 minutes rest, in cryotubes, 1:10, Bandelin Sonorex RK100, 230 V, 0.4 A, 35 kHz) for one hour (Figure 2B) (similar micrographs resulting from Sonifier 250 not shown). Sonication did not result in significant breakdown of the particles beyond what they were able to do from their standard treatment.

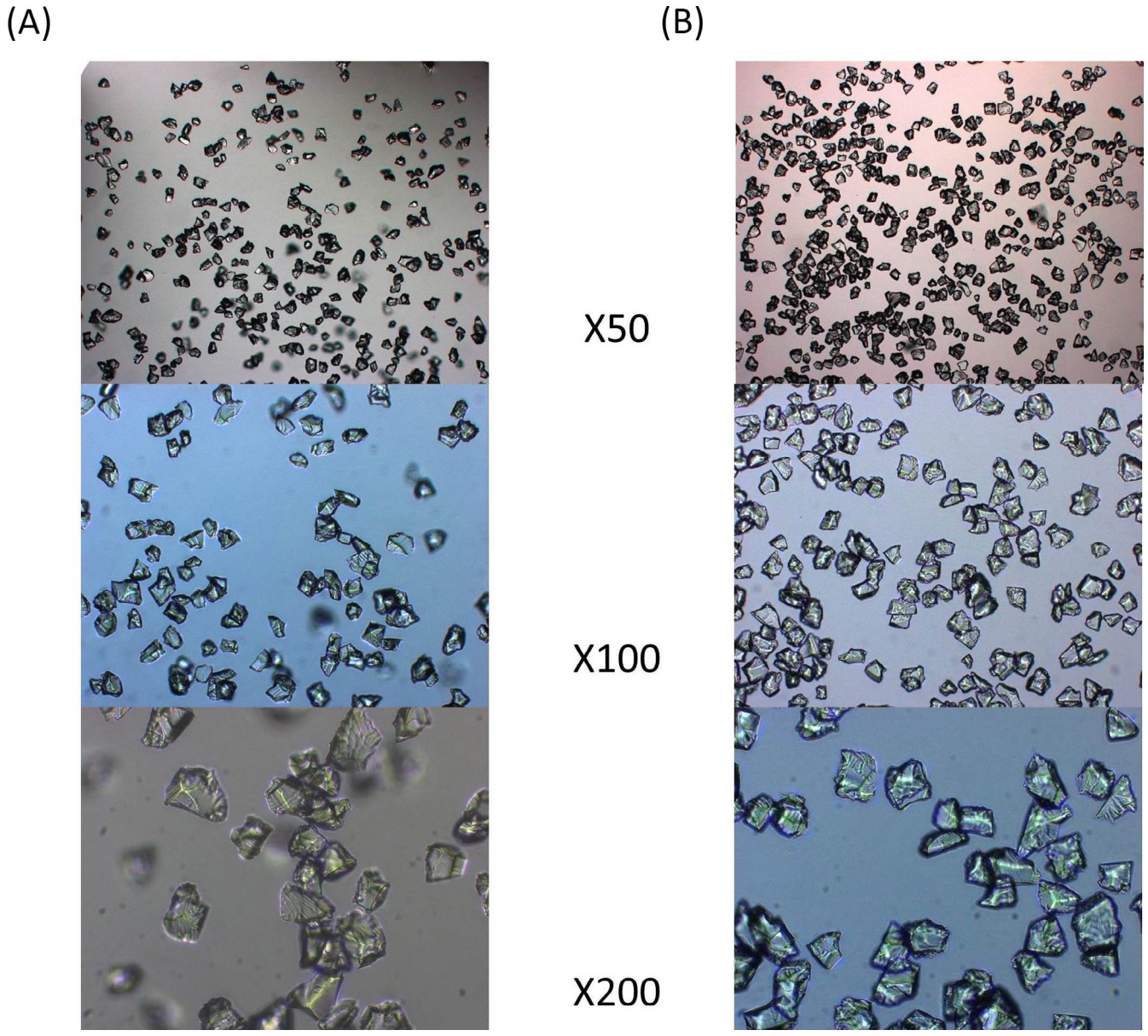

FIGURE 2 Before sonication (A) and after sonication for 1 h (B). After breaking down the particles to their smallest size, no difference in fragment size could be observed. (10 min six times, in between 5 min rest, in cryotube, 1:10, Bandelin Sonorex RK100, 230 V, 0.4 A, 35 kHz)

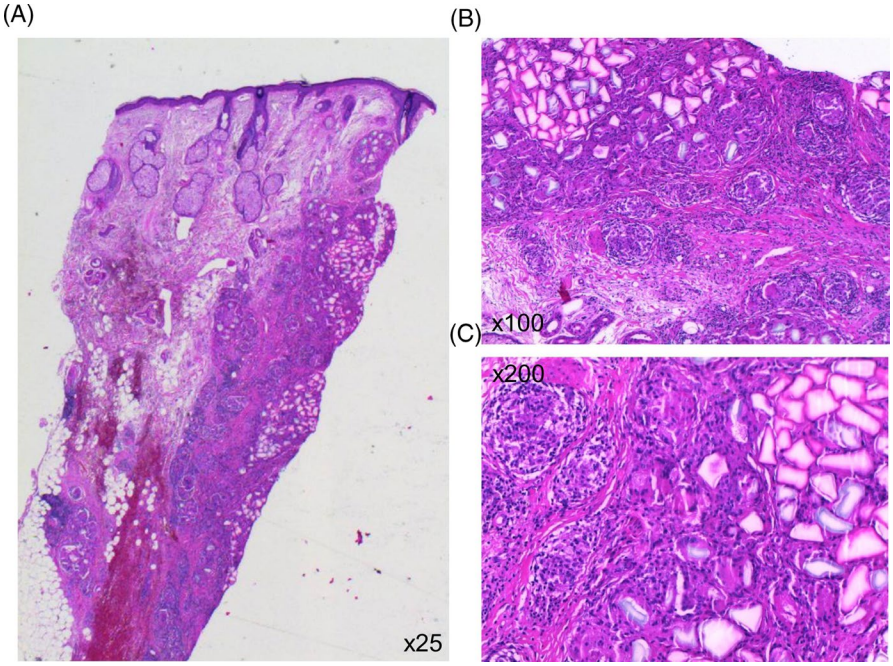

FIGURE 3 Filler granuloma, hematoxylin and eosin stain 25x (A), 100x (B), and 200x (C) magnification

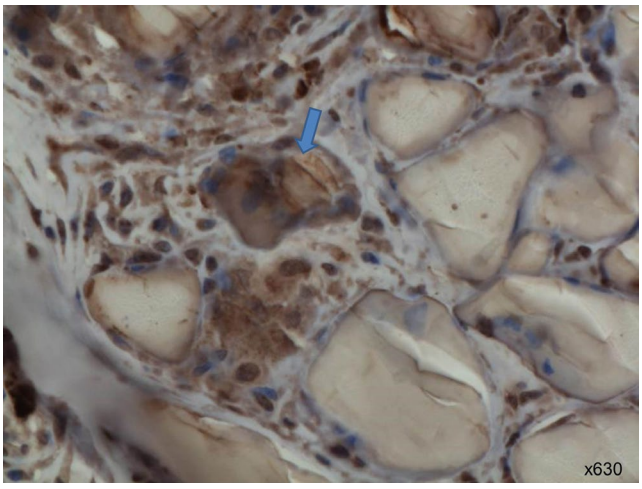

FIGURE 4 Some small filler particles are engulfed by macrophages. Filler granuloma from the patient sample: cleaved caspase-1 staining

Histologically, a nodular and diffuse granulomatous infiltration throughout the dermis and partially the subcutis was seen (Figure 3). Single and clustered vacuoles were embedded in a sclerotic stroma, suggesting a sclerosing granuloma; at higher magnification these translucent, sharply circumscribed foreign bodies corresponded to the implanted filler particles. Some smaller particles were engulfed by macrophages (Figure 4), which could not be recreated in our in vitro experiments.

In immunohistochemical analyses, no significantly increased expression of inflammasome components or products was detected. The analysis of the sensor NALP3, of the expression levels of IL-1β, IFN-γ, and of the key protein of the inflammasome complex caspase-1 revealed no significantly elevated expression (Figure 5A-I).

4 | DISCUSSION/CONCLUSION

Rejuvenation of the face using volumetric fillers has become increasingly popular since they also belong to the least invasive interventions.[15] At the same time, a significant number of patients still do suffer from complications after filler treatments from recent years, such as granuloma formation after Dermalive® application.[5] A better understanding of the adverse reactions to the acrylic hydrogel filler Dermalive®, as the product with probably highest risk of complications, could possibly help improving the current therapeutic approaches to treat the clinical signs of granulomas.[15] Furthermore, elucidating the reaction to Dermalive® could further help understand the principle of foreign body reactions to any of the injected materials today such as Sculptra®, Ellansé™, or hyaluronic acid.

One important key player in innate immunity and inflammation is the inflammasome. It is defined as a cytosolic protein complex in macrophages and neutrophil granulocytes, which detects intracellular danger-associated molecular patterns (DAMPs) of bacteria or fungi. Additionally, it can detect organic (uric acid or cholesterol crystals) and inorganic crystals (silicate dioxide or asbestos).[6,12,13]

As filler granulomas are infiltrated and surrounded by macrophages, we speculated that the semi-crystalline structure of the filler material Dermalive® might play an important role in the development of filler granulomas, as it might cause a stimulation of the inflammasome.

The activation of the inflammasome triggers several intracellular cascades which lead to the activation of caspase-1. Caspase-1 activates precursors of the pro-inflammatory molecule IL-1β. Activated IL-1β is then secreted by the macrophages and leads to an inflammatory reaction.[6,12,13] One of the important proteins in the inflammasome is NALP3, also known as cryopyrin.[16] In humans, this protein is encoded by the *NLRP3* gene (NOD-like receptor pyrin domain-containing protein 3).[17]

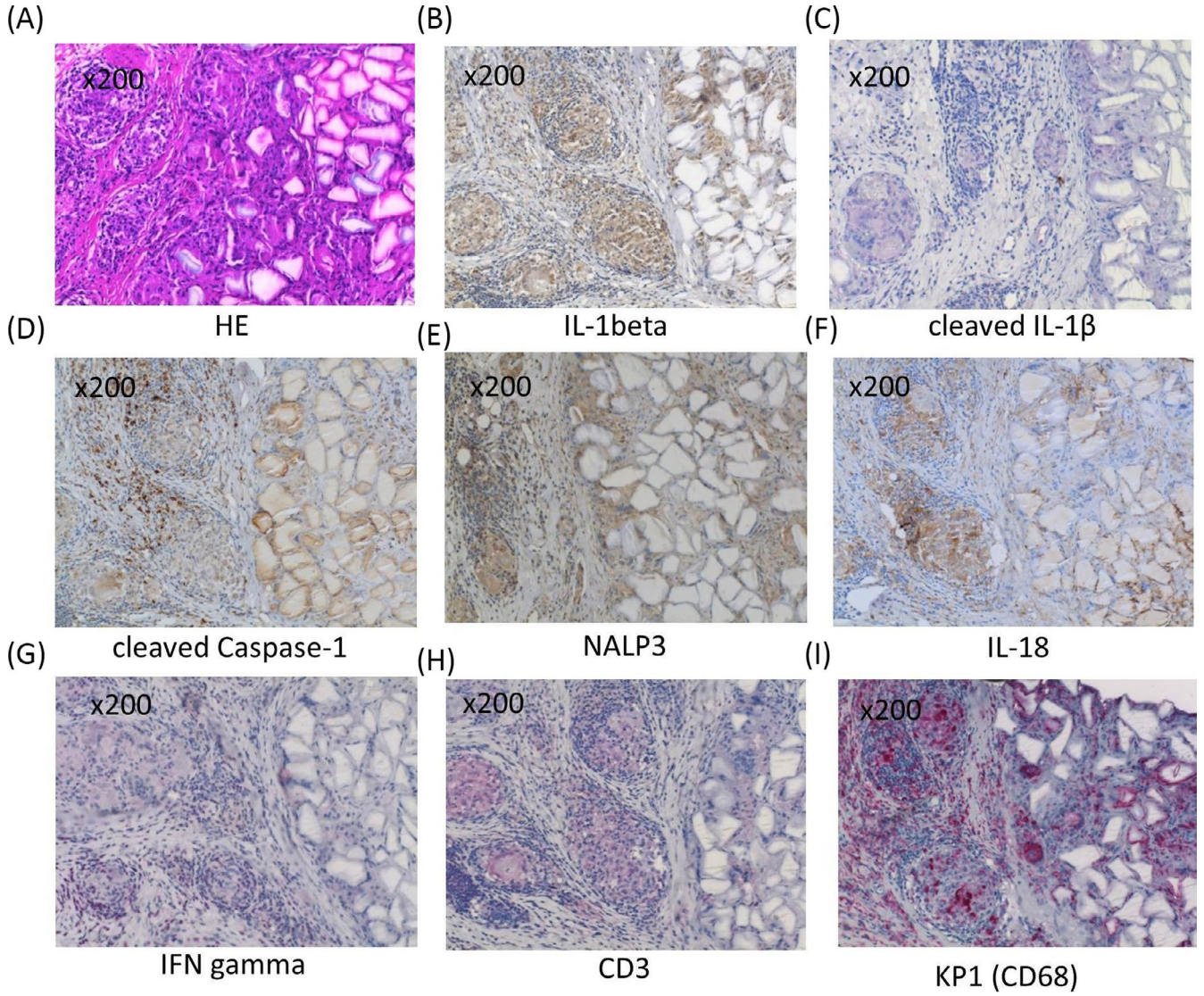

FIGURE 5 Filler granuloma, hematoxylin and eosin stain (A); filler granuloma, IL-1β (H153, sd7884, Santa Cruz) (B); filler granuloma, cleaved IL-1β (Cell signaling, #2021S) (C); filler granuloma, cleaved caspase-1 (p20,h297,sc22163, Santa Cruz) (D); filler granuloma, NALP3 (ab17267, Abcam) (E); filler granuloma, IL-18 (sc6177, Santa Cruz) (F); filler granuloma, IFN-γ (ab9657, Abcam) (G); filler granuloma, CD3 (H); and filler granuloma, KP1 (CD68) (I)

We were able to show in our work that the filler granuloma is infiltrated with and surrounded by macrophages in biopsies from all patients which is consistent with the literature.[2,18] Additionally, in histological specimens we could observe macrophages that were phagocytosing filler particles, which is a prerequisite for them to get in contact with the inflammasome. Further staining of the downstream molecules of the inflammatory cascade of the inflammasome like IL-1β and cleaved IL-1β showed no specific increase in the release of these inflammatory peptides. Neither was an increased expression of cleaved caspase-1 around the filler particles detectable. The sensor NALP3 was not significantly increased either. Also, IL-18 was detected but not significantly increased in the filler granuloma region. The infiltration of CD3 positive cells was moderate whereas the infiltration of KP1/CD68-positive cells was high.

In vitro, there was no elevated release of IL-1β or IL-8 detectable in the activated macrophages. Phagocytosis could not be observed either in cell culture experiments after up to 72 hours. A reasonable explanation would be that filler granulomas often appear years after application, probably requiring a chronic exposure of filler particles to immune cells to allow macrophage adhesion, phagocytosis of particle fragments, and in result induce inflammation.[2,14,19] Follow-up observations at later points in time could have been necessary to show changes in the activity of these cytokines. The size of the particles is also important to induce inflammation; therefore, we tried ultrasound fragmentation of the filler particles, which however, did not lead to smaller particles nor an increased inflammatory reaction of the activated macrophages. Bentkover stated that only polymer particles >20 μm in diameter are phagocytosed by polymorphonuclear cells, larger particles, like 45 to 65 μm particles as in Dermalive®, resist initial phagocytosis and are being attacked by macrophages. Also, phagocytosis is facilitated by a rough surface and sharp edges of the particles, which also increases the surface area.[20] Hence, either particle breakdown to enable phagocytosis would be required to activate cytoplasmatic inflammatory cascades or inflammation could be triggered by macrophage surface receptors, for example, via

pattern recognition receptors (PRRs) such as CD36.[21] CD36 senses polyanionic surface structures, which might form from spontaneous or enzymatic hydrolysis of the esterified acrylate residues (ethyl and 2-hydroxyethyl groups), potentially resulting in an immunogenic polyanionic particle surface over the course of months to years in vivo. However, both ways would result in inflammasome activation, which could not be observed in the histological specimens.

In conclusion, we could not show a significantly increased activation of the inflammasome, inflammasome sensors, or the subsequent cascade in filler granuloma. Neither could an increased level of IFN-γ or IL-1β be observed. However, a strong infiltration of granulocytes and macrophages could clearly be observed, which suggests a certain role of the innate immunity.

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None.

CONFLICT OF INTEREST

M Reinholz has received honoraria from MSD, Pierre Fabre, L'Oreal, Almirall, Asclepion, Lumenis, Meda/Mylan and Galderma, Leti Pharma, Sebapharma, Beiersdorf, and GSK for participation as an advisor and speaker. G G Gauglitz has received honoraria for lectures, advisory boards or consultanties from Lumenis, Merz Pharmaceuticals, Galderma, Biofrontera, Asclepion, Candela & Syneron, Pollogen, Classys, Sinclair, and MiToPharm. M V Heppt has received speaker's honoraria from Roche, Novartis, BMS, MSD, Galderma and travel support from BMS and Novartis and advisory board fees from Roche and Sanofi-Aventis. L E French has served in a function as consultant, advisory board member or clinical trials principal investigator for Abbvie, Celgene, Amgen, Galderma, Janssen-Cilag, Novartis, Pierre-Fabre, and Eli-Lilly. All other authors have no conflicts of interest to declare.

ETHICAL APPROVAL

The design and performance of our research on unidentifiable human material were ethically in accordance with the World Medical Association Declaration of Helsinki. Ethic approval (No: 17-666 UE) was obtained by the ethics committee of the medical faculty of Ludwig Maximilians University Munich.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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