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Anti-Inflammatory Activity of a Demineralized Bone Matrix: An In Vitro Pilot Study

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Abstract: Demineralized bone matrix (DBM) is commonly used for the reconstruction of bone defects. Early graft consolidation involves a transient inflammatory process. It is, however, unclear whether DBM can modulate this process. To test this possibility, we prepared acid lysates of demineralized ground cortical (DGC) and moldable demineralized fibers (MDF). Murine RAW 264.7 and primary bone marrow macrophages were exposed to acid lysates of DGC and MFD prior to provoking an inflammatory response with lipopolysaccharide (LPS). Similarly, murine ST2 mesenchymal cells were exposed to DGC and MFD with and without interleukin 1β (IL1) and TNFα. We show here that acid lysates of DGC and MFD reduced the expression of IL1 and IL6 in RAW 264.7 macrophages, as determined by RT-PCR and, for IL6, by immunoassay. This response was confirmed with primary macrophages. Likewise, desalted acid lysates exert anti-inflammatory properties on RAW 264.7 cells and in ST2 cells, the forced expression of IL6, inducible nitric oxide synthase (iNOS) and chemokine ligand 5 (CCL5) was reduced. These in vitro findings suggest that DGC and MFD lower the inflammation-induced expression of inflammatory mediators in murine cell-based bioassays.

Keywords: demineralized bone matrix; inflammation; moldable demineralized fibers; demineralized ground cortical; allografts; bone regeneration; macrophages; resolution

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1. Introduction

Demineralized bone matrix (DBM) is used to reconstruct bony defects [1], including in dental [2,3], trauma [4] and spinal surgery [5]. DBM provides an osteophilic surface where new bone can be laid, thereby allowing the ingrowth of new bone into the defect site, ideally bridging the defect [6]. The conglomerate of the DBM, the new bone, and the concomitant bone marrow undergoes remodeling and creeping substitution of the DBM, thus allowing a restitutio ad integrum of the bone and a reshaping of the anatomical contours of the former defect site [6]. Demineralized bone is derived from cadaver bone that undergoes multiple steps of processing until it is available as a ready-to-use allograft [1]. This processing pays particular attention to maintain the activity of the growth factors stored in the extracellular matrix, most of all the members of the bone morphogenetic protein (BMP) family with their unique osteoinductive properties [7,8]. DBM is thus equipped with the ability to initiate de novo bone formation at ectopic sites, which is usually tested in rodent models [9,10]. Hence, there is evidence that DBM allografts are a source of growth factors that can potentially support bone regeneration, resulting in graft consolidation in a patient.

Bone is a rich source of TGF-β [11,12]. We have recently identified TGF-β to be released into a liquid fraction from bone allografts, e.g., demineralized ground cortical (DGC) and

moldable demineralized fibers (MDF) [13]. Apart from the growth factor activity, TGF-β is thought to have anti-inflammatory functions [14]. It is therefore reasonable to assume that DGC and MDF exert an anti-inflammatory activity. Surprisingly, however, allografts are usually not tested for their potential anti-inflammatory activity [1]. It would be interesting to know if allografts exert such activity. This is because a transient local inflammation occurs during early bone regeneration [15] and, if not resolved, the chronic inflammation causes the pathological event of inflammatory osteolysis [16]. Theoretically, upon creeping substitution, DBM could potentially exert inflammation-modulating activity.

To determine the possible anti-inflammatory activity of DGC and MDF, we took the advantage of our previously established protocol to prepare acid lysates of bone [12] and dentin [17]. We then applied the lysates of DGC and MDF to our established in vitro inflammation assay. Our bioassays include murine RAW 264.7 macrophages and primary bone marrow-derived macrophages being exposed to bacterial endotoxins [18], as well as murine ST2 bone marrow-derived mesenchymal cells [19] and human gingival fibroblasts being exposed to inflammatory cytokines interleukin 1β and TNFα [20]. The modulation of the inflammatory response is measured by changes in gene expression, including IL6, and by the intensity and nuclear translocation of phosphorylated p65 [21]. IL1 as well as IL6 are lead cytokines that represent a large panel of inflammatory mediators, all of which initiate and enhance an inflammatory process that is resolved in a physiologic situation of wound healing [22] and fracture repair [15]—but, if not being resolved, becomes the main cause of tissue destruction [23]. Inducible nitric oxide synthases (iNOS) is also involved in fracture healing [24] and mesenchymal cells expressing chemokine ligand 5 (CCL5; also known as RANTES) support revascularization [25]. In this pilot study, we show in vitro data suggesting that acid lysates prepared from DGC and MDF lower the expression of inflammatory mediators.

2. Material and Methods

2.1. Preparation of Acid Lysates of DGC and MDF

Demineralized ground cortical (DGC, AlloGraft DGC, Straumann, Basel, Switzerland; 1.2 cm^3, around 0.7 g; lot IDs: 2010131-3098, 2010131-3070, 2010131-3102, 2010131-3105, 2010131-3087, 2010131-3067, 2010131-3083) and moldable demineralized fibers (MDF, OraGRAFT® Prime, LifeNet Health Europe GmbH, Vienna, Austria, 1.0 cm^3, around 0.4 g; IDs: 1814212-3196, 1814212-3144, 1814212-3165, 1814212-3193, 1814212-3158, 1814212-3178, 1814212-3134) were submerged to reach 0.1 g/mL in 1M HCl and left shaking overnight at room temperature. Acid lysates from DGC and MDF were collected through one centrifugation step at 20,000× *g* for five minutes. The pH was then neutralized with 1.0 M NaOH and filtered sterile (0.2 µm, VWR international, Radnor, PA, USA). After processing, we obtained around 10 mL and 6 mL of DGC and MDF lysates, respectively. The lysates were stored in aliquots at −20 °C. The lysates were prepared individually from each lot and experiments represent individual lots. Lysates from lots were only pooled when not reaching enough volume for a given experiment. For desalting, acid lysates were prepared with 1.0 M HCl as indicated but the pH was neutralized with 10.0 M NaOH to keep the dilution of the sample low. Then, the lysates were subjected to a PD SpinTrap G-25, a microspin column that is designed for desalting and buffer exchange of biological sample following the instructions of the manufacturer (Sigma Aldrich, St. Louis, MO, USA). The new buffer was the serum-free medium.

2.2. Murine RAW 264.7 and Bone Marrow-Derived Macrophages

RAW 264.7 macrophage cells (LGC Standards, Wesel, Germany) were expanded at 3×10^5 cells/cm^2 into 24-well plates in growth Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich), 10% fetal calf serum (Bio&Sell GmbH, Nuremberg, Germany) and antibiotics (Sigma Aldrich). Bone marrow cells were collected from the femurs and tibias of female Balb/c mice aged 6–8 weeks. Bone marrow cells were seeded at 1×10^6 cells/cm^2 into 24-well plates and grown for 7 days in αMEM (Gibco, New York, NY, USA) sup-

plemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA), antibiotics (Sigma Aldrich) and 30 ng/mL M-CSF (Prospec, Ness-Ziona, Israel). Cells were pre-exposed to 25% acid lysates from DGC and MDF for 30 min before being exposed to 100 ng/mL LPS (Escherichia coli 0111: B41; Sigma Aldrich) for 24 h [18]. To test for the involvement of TGF-β signaling, the TGF-β receptor I kinase inhibitor SB431542 (Calbiochem, Merck, Billerica, MA, USA) was added at 10 μM [13]. In another set of experiments, RAW 264.7 cells were exposed to 10–300 mM NaCl prior to the LPS challenge. Then, RT-PCR and immunoassays were performed.

2.3. ST2 Mesenchymal Stromal Cell Line and Human Gingival Fibroblasts

The ST2 mesenchymal stromal cell line was originally isolated from mouse bone marrow (RIKEN Cell Bank, Tsukuba, Japan). Tissue samples of human gingiva were harvested from the extracted third molars of three young and healthy patients who had given informed and written consent (Ethics Committee of the Medical University of Vienna; EK NR 631/2007). ST2 cells and gingival fibroblasts from pooled donors were plated in growth medium consisting of DMEM, 10% fetal calf serum (Bio&Sell GmbH) and antibiotics (Sigma Aldrich) at 30,000 cells/cm^2 into culture dishes. Cells were pre-exposed to 25% acid lysates from DGC and MDF for 30 min before being stimulated with IL1β and TNFα (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel), both at 10 ng/mL, in the serum-free medium for 24 h [20].

2.4. RT-PCR and Immunoassay

Total RNA was isolated with the ExtractMe total RNA kit (Blirt S.A., Gdańsk, Poland). Reverse transcription (RT) was performed with the LabQ FirstStrand cDNA Synthesis Kit (LabQ, Labconsulting, Vienna, Austria). Reverse transcription-polymerase chain reaction (RT-PCR) was conducted (LabQ, Labconsulting, Vienna, Austria) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences were mIL6_F GCT ACC AAA CTG GAT ATA ATC AGG A; mIL6_R CCA GGT AGC TAT GGT ACT CCA GAA; mIL1β_F AAG GGC TGC TTC CAA ACC TTT GAC; mIL1β_R ATA CTG CCT GCC TGA AGC TCT TGT; miNOS_F GGTGAAGGGACTGAGCTGTT; miNOS_R ACGTTCTCCGTTCTCTTGCAG; mCCL5_F CCTGCTGCTTTGCCTACCTC; mCCL5_R ACACACTTGGCGGTTCCTTC; mGAPDH_F AAC TTT GGC ATT GTC GAA CG; mGAPDH_R GGA TGC AGG GAT GAT GTT CT; hIL6_F GAA AGG AGA CAT GTA ACA AGA GT; hIL6_R GAT TTT CAC CAG GCA AGT CT; hIL8_F AAC TTC TCC ACA ACC CTC TG; hIL8_R TTGGCAGC CTTCCTGATTTC; hGAPDH_F AAG CCA CAT CGC TC; hGAPDH_R AGA CAC GCC CAA TAC GAC CAA ATC C. For primers on proliferation markers, see Supplementary Table S1. The mRNA levels were calculated by normalizing to the housekeeping gene GAPDH using the ΔΔCt method. The immunoassay was performed with the mouse IL6 and human IL8 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.5. Immunofluorescence

RAW 264.7 macrophages were plated in growth medium onto Millicell® EZ slides (Merck KGaA, Darmstadt, Germany). The following day, cells were treated with serum-free medium overnight. The next day, cells were exposed to 25% DGC and MDF followed by 100 ng/mL LPS for 10 min each. Cells were then fixed in paraformaldehyde and blocked in 5% bovine serum albumin (BSA) and 0.3% Triton X-100 in phosphate-buffered saline (PBS) at room temperature, after which permeabilization with 0.1% Triton X-100 took place. The cells were then incubated with rabbit anti-human NFκB p65 (#8242; Cell Signaling Technology, Cambridge, UK) at 4 °C overnight. Detection was performed with the goat anti-rabbit Alexa 488 secondary antibody (CS-4412, Cell Signaling Technology). Finally, cells were washed and mounted onto glass slides. Images were captured under a fluorescent microscope (Axio Imager M2, Carl Zeiss AG, Oberkochen, Germany).

2.6. Statistical Analysis

Statistical analysis comparing the inflammation group alone versus the DGC and MDF groups was based on a Friedmann test using GraphPad Software (Version 8, San Diego, CA, USA). To compare the DGC and MDF groups, a Wilcoxon matched-pairs signed rank test was performed that was in neither case significant and, thus, the *p*-values are not shown.

3. Results

3.1. Acid Lysates of DGC and MDF Do Not Affect the Viability of RAW 264.7 Macrophages

We first determined whether or not DGC and MDF can change the viability of the RAW 264.7 cells. The RAW 264.7 cells were grown in the presence of 12, 25 and 50% allograft lysates for 24 h followed by a classical MTT viability assay. As indicated in Supplementary Figure S1, there was no considerable change in cell viability. Consistently, Figure 1 shows that RAW 264.7 cells exposed to LPS undergo the expected morphological changes typical for dendritic cells [26]. No detachment or other obvious signs of cell death occurred in the presence of 25% desalted DGC and MDF.

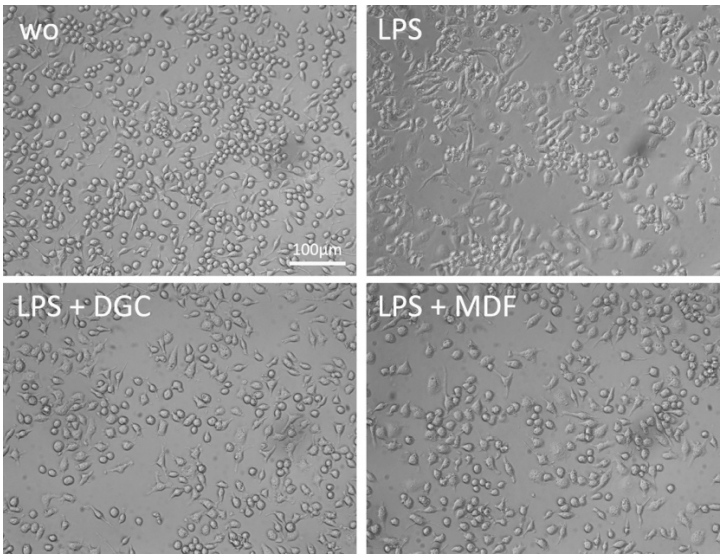

Figure 1. Morphological appearance of LPS-exposed RAW 264.7 macrophages in the presence of desalted DGC and MDF.

RAW 264.7 macrophages were exposed to 25% acid lysates of desalted DGC and MDF for 30 min followed by 100 ng/mL LPS for 24 h. No detachment or any other obvious signs of cell death were noticed when LPS-exposed RAW 264.7 macrophages were grown in the presence of desalted DGC and MDF.

3.2. Acid Lysates of DGC and MDF Reduce the Inflammatory Response of Macrophages

Next, we investigated whether DGC and MDF can modulate the inflammatory response of macrophages. To this end, we exposed RAW 264.7 cells to the allograft lysates before the expression of pro-inflammatory cytokines was induced by LPS. DGC and MDF caused a reduction in the LPS-induced expression of IL1 and IL6, which was confirmed at the protein levels by IL6 immunoassay (Figure 2). Blocking of the TGF-β receptor type I kinase with SB431542 failed to reverse the anti-inflammatory activity of the lysates (Supplementary Table S2). LPS initiated the nuclear translocation of p65 in RAW 264.7 cells, which was, however, only moderately reduced by DGC and MDF (Figure 3). Strong support for the anti-inflammatory activity of DGC and MDF comes from experiments with primary macrophages where DGC and MDF consistently reduced the expression of IL1 and IL6 of LPS-treated cells (Figure 4).

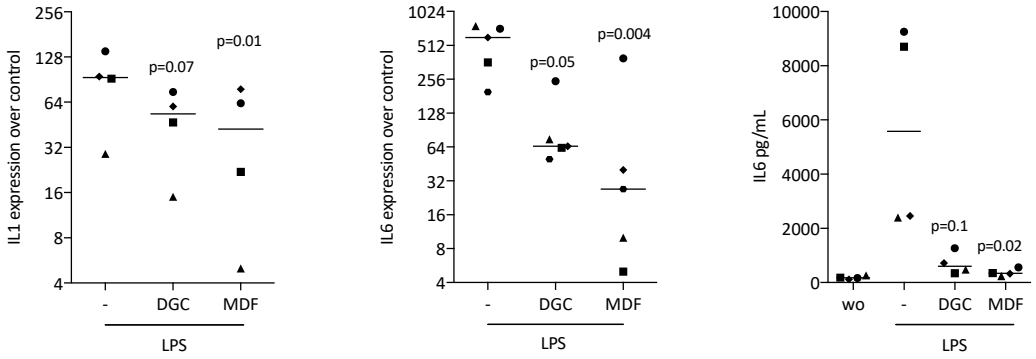

Figure 2. DGC and MDF reduced IL1 and IL6 in LPS-stimulated RAW 264.7 macrophages.

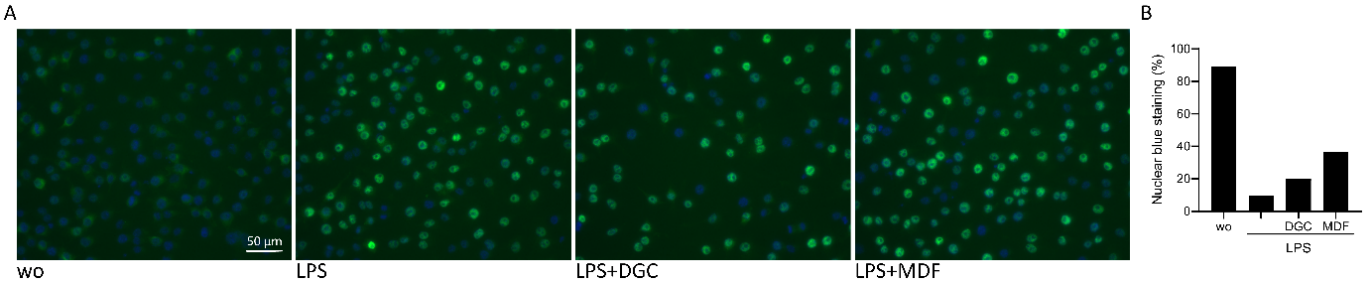

Figure 3. DGC and MDF weakly lowered p65 nuclear staining in LPS-stimulated RAW 264.7. (**A**) Immunostaining revealed the green fluorescence signals obtained with the p65 antibody. Nuclear staining with DAPI appears blue. (**B**) Mean signal intensity of nuclear staining by obtained by ImageJ software.

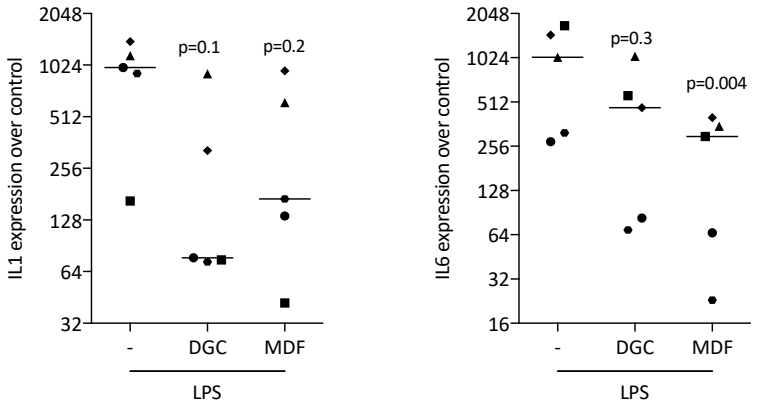

Figure 4. DGC and MDF reduced IL1 and IL6 in LPS-stimulated primary macrophages.

RAW 264.7 macrophages were exposed to 25% acid lysates of DGC and MDF for 30 min followed by 100 ng/mL LPS for 24 h. Data show the relative expression changes normalized to the untreated cells. In addition, the IL6 ELISA data are shown. The experiments were performed four times, represented by a unique symbol for each replicate (N = 4). The statistical analysis was based on a Friedmann test and *p*-values are indicated. The Wilcoxon matched-pairs signed rank test revealed $p > 0.6$ when comparing DGC and MDF alone (not shown).

RAW 264.7 were exposed to LPS with or without DGC and MDF. WO means without and represents unstimulated cells.

The primary macrophages were exposed to 25% acid lysates of DGC and MDF for 10 min followed by 100 ng/mL LPS for 24 h. Data show the relative expression changes normalized to the untreated cells. The experiments were performed five times, represented by a unique symbol for each replicate (N = 5). The statistical analysis was based on a

Friedmann test and *p*-values are indicated. The Wilcoxon matched-pairs signed rank test revealed $p > 0.6$ for IL1 and $p = 0.063$ for IL6 when comparing DGC and MDF alone (not shown).

3.3. Desalted Acid Lysates Reduce the Inflammatory Response of Macrophages

Considering that the salt generated during the neutralization process may have suppressed the LPS-induced cytokine expression [27], we included a dose–response experiment with NaCl being added to the culture medium. Indeed, adding 100 mM but not 30 mM NaCl greatly diminished the LPS-induced expression of IL1 and IL6 by the RAW 264.7 cells (Table 1). Therefore, DGC and MDF containing NaCl produced by pH neutralization underwent desalting and buffer exchange before testing for a potential anti-inflammatory activity. As indicated in Figure 5, desalted DGC and MDF caused a reduction in the LPS-induced expression of IL1 and IL6. Thus, even though NaCl alone can reduce the expression of cytokines, the same was true for the desalted and buffer-exchanged preparations of DGC and MDF.

Table 1. Additional NaCl reduced the expression of IL1 and IL6 in RAW 264.7 macrophages.

NaCl Concentration	LPS + 0 mM	LPS + 10 mM	LPS + 30 mM	LPS + 100 mM
Experiment 1 (IL1/IL6)	170.7/422.1	369.0/521.3	807.2/594.4	134.5/10.5
Experiment 2 (IL1/IL6)	157.6/849.0	190.3/797.4	233.4/1065.4	7.9/5.5

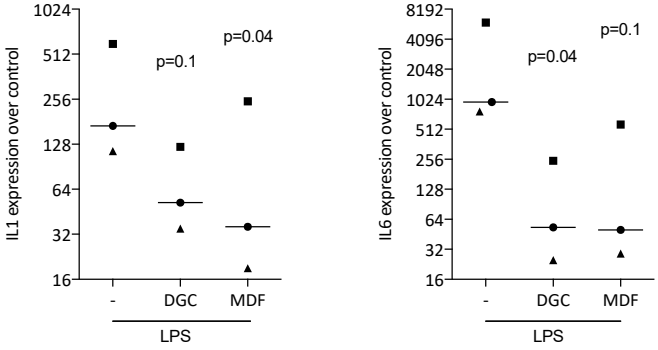

Figure 5. Desalted acid lysates of DGC and MDF reduced the inflammatory response of LPS-stimulated RAW 264.7 macrophages.

RAW 264.7 macrophages were exposed to the indicated concentrations of NaCl being added to the culture medium for 30 min followed by 100 ng/mL LPS for 24 h. Data show the relative expression changes normalized to the untreated cells. The experiments were performed two times, indicated as experiment 1 and 2.

RAW 264.7 macrophages were exposed to 25% concentrations of desalted and buffer-exchanged acid lysates of DGC and MDF for 30 min followed by 100 ng/mL LPS for 24 h. Data show the relative expression changes normalized to the untreated cells. The experiments were performed three times, represented by a unique symbol for each replicate. Statistical analysis was based on a Friedmann test and *p*-values are indicated. The Wilcoxon matched-pairs signed rank test revealed $p > 0.99$ and $p = 0.5$ for IL1 and IL6, respectively, when comparing DGC and MDF alone (not shown).

To rule out that the anti-inflammatory activity of the allografts was caused by the adsorption of LPS to the acid-soluble extracellular matrix, we pre-exposed the RAW 264.7 macrophages to LPS and then exposed them to the desalted DGC and MDF, and vice versa. In both settings, we saw an equivalent inhibition of IL1 (72.8% $\pm$ 4.7 versus 67.5% $\pm$ 12.1) and IL6 (96.2% $\pm$ 0.4 versus 95.9% $\pm$ 1.1) expression by DGC. An equivalent

inhibition of IL1 (80.7% ± 3.2 versus 79.2% ± 3.3) and IL6 (94.3% ± 2.3 versus 95.5% ± 0.6) expression was observed with MDF (data not shown).

3.4. Acid Lysates of DGC and MDF Reduce the Inflammatory Response of ST2 Cells

Finally, we tested whether or not DGC and MDF can dampen the inflammatory response of ST2 cells and gingival fibroblasts. In ST2 cells exposed to IL1β and TNFα, DGC and MDF decreased the expression of IL6, iNOS and CCL5 (Figure 6). In gingival fibroblasts exposed to IL1β and TNFα, DGC and MDF only moderately reduced the expression of IL6 and IL8 (Supplementary Figure S2). Moreover, DGC and MDF had no impact on the expression of the proliferation marker genes Ki67, PCNA and CCND1 in the gingival fibroblasts (data not shown). Thus, DGC and MDF can reduce the expression of IL6 and other inflammatory mediators in murine mesenchymal ST2 cells.

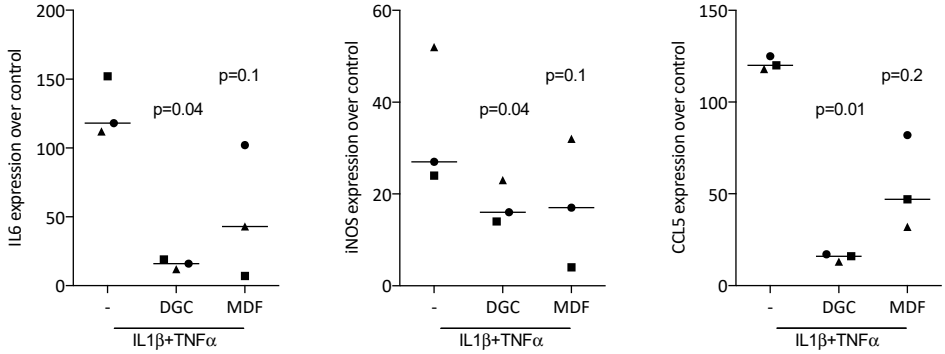

Figure 6. Desalted DGC and MDF reduced the expression of IL6, iNOS and CCL5 in ST2 cells.

ST2 cells were exposed to 25% desalted acid lysates of DGC and MDF for 30 min followed by 10 ng/mL of IL1β and TNFα for 24 h. Data show the relative expression changes normalized to the untreated cells. The experiments were performed three times, represented by a unique symbol for each replicate (N = 3). Statistical analysis was based on a Friedmann test and *p*-values are indicated. The Wilcoxon matched-pairs signed rank test revealed $p = 0.5$, $p = 0.2$ and $p > 0.9$ for IL6, iNOS and CCL5, respectively, when comparing DGC and MDF alone (not shown).

4. Discussion

Demineralized bone matrix [1] may have biological activities that remain overshadowed by the impressive osteoinductive properties when transplanted ectopically in a rodent model [9,10]. In a clinical scenario, however, demineralized freeze-dried bone allografts failed to exert the hallmark signs of osteoinductivity [28]. Moreover, demineralized teeth are proposed as grafts for bone reconstruction [17,29]. DBM research is closely related to the discovery of the BMPs [1,7,30], yet attempts to identify other growth factors that survive the demineralization process are minor [13,31,32]. Surprisingly, however, even though the early stages of graft consolidation occur in a catabolic inflammatory environment [15], we lack research on whether DBM might influence the process of inflammation. We therefore tested the potential anti-inflammatory properties of acid lysates prepared from DGC and MDF based on our established bioassays.

The main finding of the present research was that acid lysates from granules and fibers reduced the inflammatory response of RAW 264.7 macrophages, particularly IL1 and IL6 expression. This anti-inflammatory activity was not restricted to RAW 264.7 macrophages as the LPS-induced expression of inflammatory cytokines in the primary macrophages was also diminished by DGC and MDF. Care should be taken when interpreting these findings because NaCl generated by the neutralization of HCl might have anti-inflammatory properties; however, it needs around 200 mM NaCl to cause an M1 to M2 shift of primary human macrophages [27]. We confirmed that adding 300 mM NaCl to the culture medium is toxic to RAW 264.7 macrophages and observed an anti-inflammatory activity by adding 100 mM

NaCl to the culture medium. To rule out that the anti-inflammatory activity of DGC and MDF is an artefact caused by the disbalanced salt concentration of the culture medium, the lysates were subjected to desalting and buffer exchange for serum-free medium. In support of the overall conclusion, desalted and buffer-exchanged DGC and MDF greatly reduced the LPS-induced expression of IL1 and IL6 in RAW 264.7 macrophages.

To rule out that DGC and MDF bind to LPS and thereby neutralize its activity, we first exposed the RAW 264.7 macrophages to LPS before adding the lysates. In this setting, the desalted lysates of both allografts had an anti-inflammatory activity with respect to the expression of IL1 and IL6 in the RAW 264.7 macrophages. To further rule out this possibility, we implemented the ST2 murine mesenchymal cell line where IL1β and TNFα can provoke an inflammatory response [19]. We show here that with ST2 cells, desalted DGC and MDF significantly attenuated the expression of IL6, iNOS and CCL5. Together, these findings suggest that the anti-inflammatory activity of the allograft lysates cannot be simply blamed on the neutralization of LPS. Moreover, these data extend our observation obtained with macrophages towards mesenchymal cells. Surprisingly, however, the inflammatory response of human gingival fibroblast to IL1β and TNFα was only moderately reduced by DGC and MDF. These findings suggest that the murine cells are potentially more sensitive than human cells to lysates of DGC and MDF in their activity in terms of reducing the expression of inflammatory mediators.

The present study has limitations. Even though we describe here an anti-inflammatory effect of acid lysates of DGC and MDF in macrophages and ST2 cells, many questions remain to be answered. First, we have not yet identified a specific molecule in DGC and MDF that is responsible for the anti-inflammatory activity. TGF-β signaling can be ruled out because blocking of the TGF-β receptor type I kinase with SB431542 failed to reverse the anti-inflammatory activity. Future studies should, therefore, include a size fractionation of the acid lysate to identify the molecular weight of the target molecule, followed by a proteomic analysis to identify possible candidates. However, this approach requires a high sample volume, which was not available when conducting the present study. Second, and consequently, the signaling cascade that leads to the reduction in the inflammatory response needs further refinement, and our data, which show only a moderate blocking of LPS-induced p65 nuclear translocation in RAW 264.7 cells, are not convincing. Third, we did not include allografts from other manufacturers, which is a limitation because each provider has slightly different protocols to prepare demineralized bone. Fourth, the reason why murine macrophages and ST2 cells, but not human gingival fibroblasts show this response remains enigmatic. Finally, we cannot tell if the anti-inflammatory activity we observed in vitro translates to in vivo, and if yes, the overall biological and clinical consequences need to be discovered in future studies. The present research should, therefore, be considered a preliminary screening approach showing that acid lysates of allografts can dampen the simulated inflammatory response of murine macrophages and mesenchymal cells under in vitro conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12020876/s1, Figure S1: DGC and MDF maintain the viability of RAW 264.7 macrophages; Figure S2: DGC and MDF moderately reduce the expression of IL6 and IL8 in IL1β and TNFα-stimulated gingival fibroblasts; Table S1: Primer sequences of proliferation marker genes; Table S2: Blocking of the TGF-β receptor type I kinase with SB431542 failed to reverse the anti-inflammatory activity of the DGC lysate.

Author Contributions: Conceptualization, L.P. and R.G.; data curation, L.P.; formal analysis, L.P. and R.G.; methodology, L.P., A.O., A.O.A. and Z.K.; project administration, L.P., A.O., A.O.A. and J.N.; supervision, L.P. and R.G.; validation, L.P., J.N. and Z.K.; visualization, L.P.; writing—original draft, L.P. and R.G.; writing—review and editing, L.P., A.O., A.O.A., J.N., Z.K. and R.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Medical University of Vienna (EK NR 631/2007).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data are available on demand.

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Conflicts of Interest: The authors declare no conflict of interest.

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