

Anti-inflammatory properties of S53P4 bioactive glass implant material

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ABSTRACT

Objectives: To assess whether the dissolution products of S53P4 bioactive glass (BG) affect cellular response of macrophages and clinically relevant peri-implant cell populations to dental implant particles *in vitro*. Cells chosen were human gingival fibroblasts (HGFs), osteoblasts and bone marrow derived stromal cells (HBMSCs). **Methods:** Melt-derived S53P4 bioactive glass were prepared. HGFs, Saos-2 human osteoblastic cell line, HBMSCs and macrophages, derived from THP-1 human monocytic cell line, were cultured in the presence of particles from commercially pure titanium (Ti-CP4), grade 5 titanium alloy (Ti-6Al-4V), titanium-zirconium alloy (Ti-15Zr) or zirconia (Zr) (with respective diameters of 34.1 ± 3.8 , 33.3 ± 4.4 , 97.8 ± 8.2 and 71.3 ± 6.1 μm) with or without S53P4 dissolution products (conditioned media contained 327.30 ± 2.01 ppm Ca, 51.34 ± 0.41 ppm P and 61.48 ± 1.17 ppm Si, pH 8.01 ± 0.21). Inflammatory and macrophage polarisation markers including TNF- α , IL-1, IL-6 and CD206 were quantified using enzyme-linked immunosorbent assay (ELISA).

Results: The presence of Ti-6Al-4V implant particles significantly induced the expression of pro-inflammatory markers in all tested cell types. S53P4 BG dissolution products regressed the particle induced up-regulation of pro-inflammatory markers and, appeared to suppress M1 macrophage polarisation.

Conclusions: Implant particles, Ti-6Al-4V in particular, resulted in significant inflammatory responses from cells. S53P4 BG may possess anti-inflammatory properties and potentially mediate macrophage polarisation behaviour.

Clinical Significance: The findings highlight that the use and benefits of BG is a promising field of study. Authors believe more collective efforts are required to fully understand the reliability, efficiency and exact mechanisms of action of BG in the search for new generation of treatment modalities in dentistry.

Introduction

Bioactive glass (BG) is a widely used bone graft material for the regeneration of bone defects caused by trauma or various diseases such as osteoporosis or tumour removal [1]. Bone graft substitutes made of glass compositions known as 45S5 (45 wt% SiO₂, 24.5 wt% CaO, 24.5 wt% Na₂O and 6.0 wt% P₂O₅) and S53P4 (53 wt% SiO₂, 23 wt% Na₂O, 20 wt% CaO and 4 wt% P₂O₅) are FDA approved devices that have been investigated in applications such as orthopaedic and dental applications [2–4]. The S53P4 composition was chosen due to its low substitution (degradation) rate, and its reported antibacterial properties, which is important in implant dentistry for long term stability of augmented sites. For metallic dental implants, a common issue that has deleterious impact on implant failure is peri-implantitis, which is characterised by

inflammation in the peri-implant mucosa due to the release of metallic particles and ions from the implants [5,6].

Metallic ions and particles of Grade 5 titanium alloy (Ti-6Al-4V) implants in particular have been reported both *in vitro* and in animal models to induce toxicity and marked upregulation of pro-inflammatory markers such as interleukin 1 beta (IL-1 β), IL-6 and tumour necrosis factor alpha (TNF- α), leading to inflammation-induced osteoclastogenesis and periprosthetic osteolysis [7–9]. Dissolution of BG results in localised increased pH, which contributes to their anti-microbial properties [1,10]. However, the effects of BG on peri-implant cell populations and inflammatory cells remain unclear. Previous studies reported that in the presence of particulate 45S5 BG, human macrophages secreted lower pro-inflammatory cytokine such as IL-6 and TNF- α when compared to zinc phosphate glasses, suggesting BG might

be of clinical use in conditions associated with inflammation [11,12].

Our hypothesis is that S53P4 BG also possess anti-inflammatory effects on not only macrophages cytokine secretion and polarisation, but also peri-implant cell populations such as gingival fibroblasts, osteoblasts and bone marrow derived stromal cells.

Materials and methods

All chemicals and reagents were purchased from Merck Life Science (Dorset, UK) or ThermoFisher Scientific (Paisley, UK). Ti-CP4 (commercial pure Ti) and Ti-6Al-4V (grade 5 Ti alloy) particles (from Carpenter Additive, Widnes, UK) were gifted by Prof Jonathan Jeffers. Straumann® (Basel, Switzerland) supplied Roxolid® (a proprietary alloy composed of ~15% zirconium and ~85% titanium, Ti-15Zr) and Zr. The diameters of Ti-CP4, Ti-6Al-4 V, Ti-15Zr and Zr were 34.1 ± 3.8 , 33.3 ± 4.4 , 97.8 ± 8.2 and 71.3 ± 6.1 μm respectively [13].

BG synthesis and dissolution product preparation

Melt-derived S53P4 BG with composition of 53.8 mol% SiO_2 , 21.8 mol% CaO , 22.7 mol% Na_2O and 1.7 mol% P_2O_5 were prepared. A mixture of raw materials containing high purity silica (SiO_2), calcium carbonate (CaCO_3), sodium carbonate (Na_2CO_3) and phosphorous pentoxide (P_2O_5) was melted at 1400°C for 1.5 h in a Pt-5%Au crucible. The melt was quenched into deionised water. The frit was collected and dried at 100°C . Frit was ball-milled and sieved to yield particles with diameters less than $32 \mu\text{m}$. Dissolution products were prepared by submerging S53P5 BG at a ratio of 75 mg to 50 ml in relevant cell culture medium for 72 h on an orbital roller at 37°C . Dissolution products were filter sterilised through $0.2 \mu\text{m}$ membrane syringe filters prior to use in cell culture. The concentrations of calcium, silicate and phosphorus were determined using a Thermo Scientific iCaP 6300 Duo inductively coupled plasma–optical emission spectrometer (ICP–OES).

Cell culture

Human gingival fibroblasts (HGFs, PCS-201–018™, ATCC, UK), Saos-2 human osteoblastic cell line (HTB-85™, ATCC, UK) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) P-S (100 unit.mL⁻¹ penicillin and 100 $\mu\text{g.mL}^{-1}$ streptomycin). Human bone marrow derived stromal cells (HBMSCs, 2M-302, Lonza, UK) were cultured in Minimum Essential Medium (MEM) with 10% (v/v) FBS and 1% (v/v) P-S.

THP-1 human monocytic cell line (88,081,201, Merck Life Science, U.K.) was cultured in Roswell Park Memorial Institute medium (RPMI-1640) with 10% (v/v) non-heat inactivated FBS and 50 pM β -mercaptoethanol (BME). THP-1 monocytic cells were differentiated into macrophages by 24 h incubation with 150 nM phorbol 12-myristate 13-acetate (PMA) and 24 h incubation with RPMI-1640.

HGFs, Saos-2, HBMSCs and THP-1 derived macrophages were cultured in the presence of Ti-CP4 (commercially pure titanium), Ti-6Al-4 V (grade 5 titanium alloy), Ti-15Zr (titanium-zirconium alloy) or Zr (zirconia) particles at a ratio of 1.5 mg to 1 mL medium with or without S53P4 dissolution products for 24 h.

Immunohistochemical staining

2.5×10^4 THP-1 cells were seeded and differentiated into macrophages in ibidi® microscopy μ -Dish (Thistle Scientific, Glasgow, UK) as described above. Undifferentiated THP-1 cells were fixated on microscopy μ -Dish by drying 100 μL PBS containing 2.5×10^4 cells. Samples were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline). Following wash and blocking with 1% (w/v) BSA (bovine serum albumin) in PBS, samples were incubated at 4°C overnight with following primary antisera from ThermoFisher Scientific (Paisley, UK): CD14 (MA5–35200, 1:100 dilution), CD36 (PA1–16813, 1:100 dilution) and

CD68 (PA5–32330, 1:100 dilution). This was followed by hour-long incubation with Alexa Fluor® 488-conjugated secondary antibody (1:1000 dilution). All samples were counter-stained with $0.1 \mu\text{g.mL}^{-1}$ DAPI and imaged under Leica SP5 confocal microscope.

Enzyme-linked immunosorbent assay (ELISA)

The release of cytokines of interest in culture medium was quantified using ELISA kits following manufacturers' instructions. The following ELISA kits were used: TNF- α (ADI-902–099, Enzo Life Sciences, UK), IL-1 β (ADI-900–130, Enzo Life Sciences, UK), IL-6 (ENZ-KIT178–0001, Enzo Life Sciences, UK) and mannose receptor (MMR/CD206, ThermoFisher Scientific, Paisley, UK).

Statistical analysis

Results were presented as mean \pm S.D ($n = 3$). Non-parametric test with Kruskal-Wallis test (for multiple groups) were performed using GraphPad Prism. Results were deemed significant if the probability of occurrence by random chance alone was less than 5%.

Results

Grade 5 titanium alloy (Ti-6Al-4V) implant particles significantly induced the secretion of TNF- α and IL-1 cytokines in HGFs, Saos-2 and HBMSCs as well as IL-6 cytokines in Saos-2 in comparison to basal and other Ti based alloys (Fig. 1). Ti-CP4, Ti-15Zr and Zr implant particles appeared to affect certain pro-inflammatory markers in certain cell type, albeit not statistically significant. The presence of S53P4 dissolution products reduced the elevated level of all three pro-inflammatory markers, induced by Ti-6Al-4V implant particles, to a level comparable to basal control.

Human THP-1 monocytic cells were differentiated into macrophages using 150 nM PMA. Suspension THP-1 cells became adherent and, the expression of CD14 decreased while CD36 and CD68 increased, confirming the macrophage formation (Fig. 2). In the presence of Ti-6Al-4V implant particles, typical pro-inflammatory/M1 marker TNF- α was significantly increased (Fig. 3a) while anti-inflammatory/M2 marker CD206 was significantly decreased (Fig. 3b). The addition of S53P4 dissolution product suppressed the elevation of TNF- α and reduction of CD206. All other implant particles did not appear to affect macrophage polarisation. The mean diameters of Ti-CP4, Ti-6Al-4V, Ti-15Zr and Zr were 34.1 ± 3.8 , 33.3 ± 4.4 , 97.8 ± 8.2 and 71.3 ± 6.1 μm respectively. The mean values for elemental concentrations in the conditioned media containing the dissolution products of S53P4 BGs were 327.30 ± 2.01 ppm Ca, 51.34 ± 0.41 ppm P and 61.48 ± 1.17 ppm Si (pH 8.01 ± 0.21).

Discussion

While the applications of BGs in osteogenesis, angiogenesis and anti-microbial activities have been widely reported [1,14], studies on the anti-inflammatory effects of BGs and the mechanism through which BGs interact with inflammatory and/or repairing cells remain sparse and focused mainly on original 45S5 BG. In wound healing, *in vitro* and *in vivo* experiments have shown that the ionic products of 45S5 BG dissolution led to macrophage activation to the M2 phenotype as well as the secretion of anti-inflammatory cytokines, which in turn resulted in the upregulation in angiogenic growth factor expression in endothelial cells [15]. In dentistry, significant reduction in gingival bleeding and supra-gingival plaque formation can be achieved by a toothpaste containing NovaMin (fine 45S5 particulates with a median size less than $20 \mu\text{m}$) [16], while topical application of 45S5 BG reduced signs of gingival inflammation in human subjects [17].

Here, we demonstrate that the presence of implant particles, in particular, grade 5 titanium alloy (Ti-6Al-4V) particles, can significantly induce the expression of pro-inflammatory markers such as TNF- α , IL-1

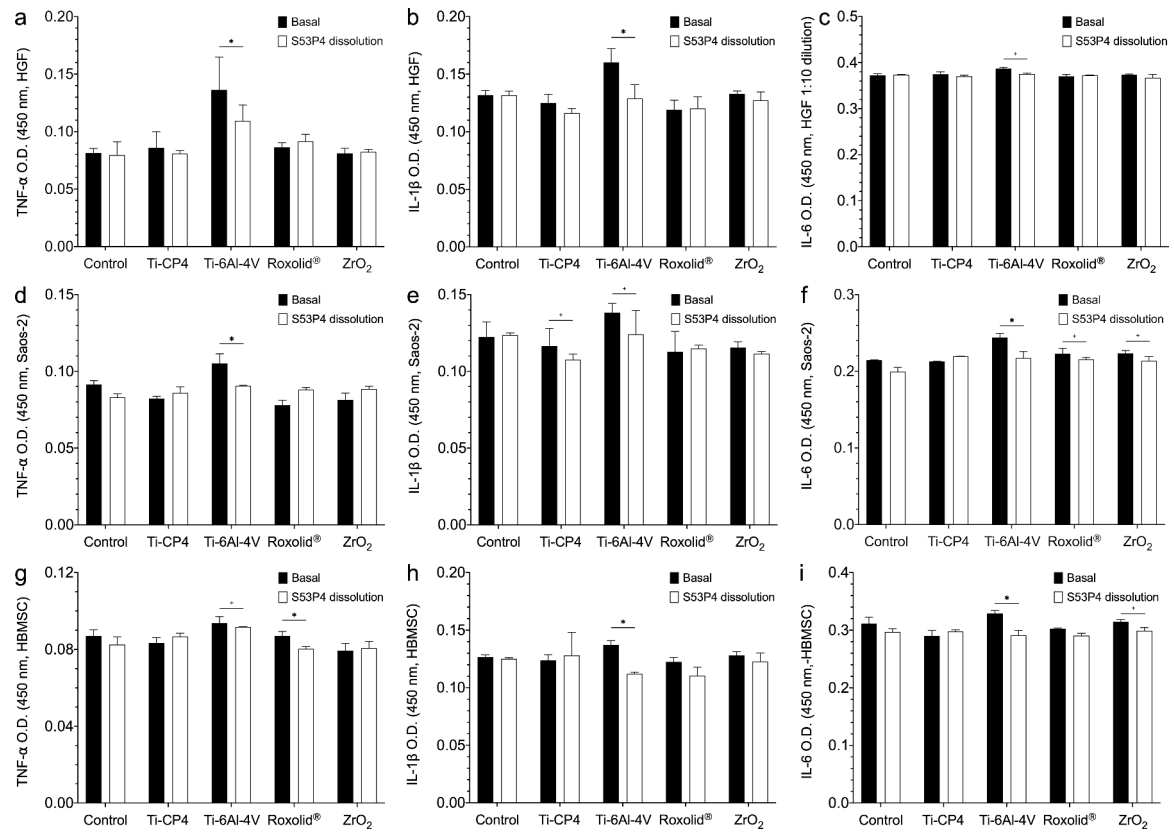


Fig. 1. Expression of inflammatory cytokines TNF-α (a, d, g), IL-1 (b, e, h) and IL-6 (c, f, i) measured by ELISA, secreted by cells on exposure to dental implant wear particles (Ti-CP4, Ti-6Al-4V, Roxolid, ZrO₂) for 24 h with and without S53P4 dissolution products: human gingival fibroblasts (HGFs) (a-c), Saos-2 osteoblastic cell line (d-f) and bone marrow derived stromal cells (BMSC) (g-i) were measured. * $p < 0.05$ and + $0.05 < p < 0.1$.

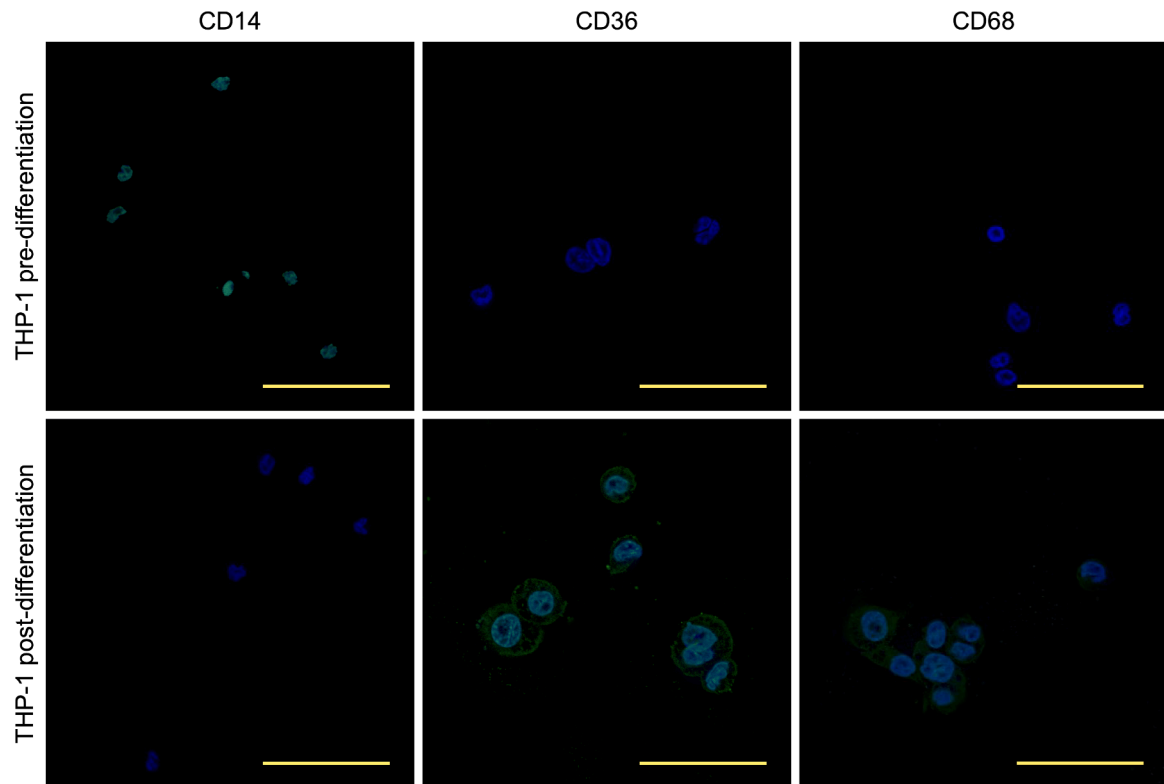


Fig. 2. Macrophage differentiation from THP-1 monocytic cells. Macrophage differentiation was achieved by 24 h incubation with 150 nM PMA followed by 24 h incubation with basal RPMI. Immunohistochemical labelling of CD14, CD36 or CD68 markers (green) were counter stained with DAPI (blue). Scale bar = 50 μm.

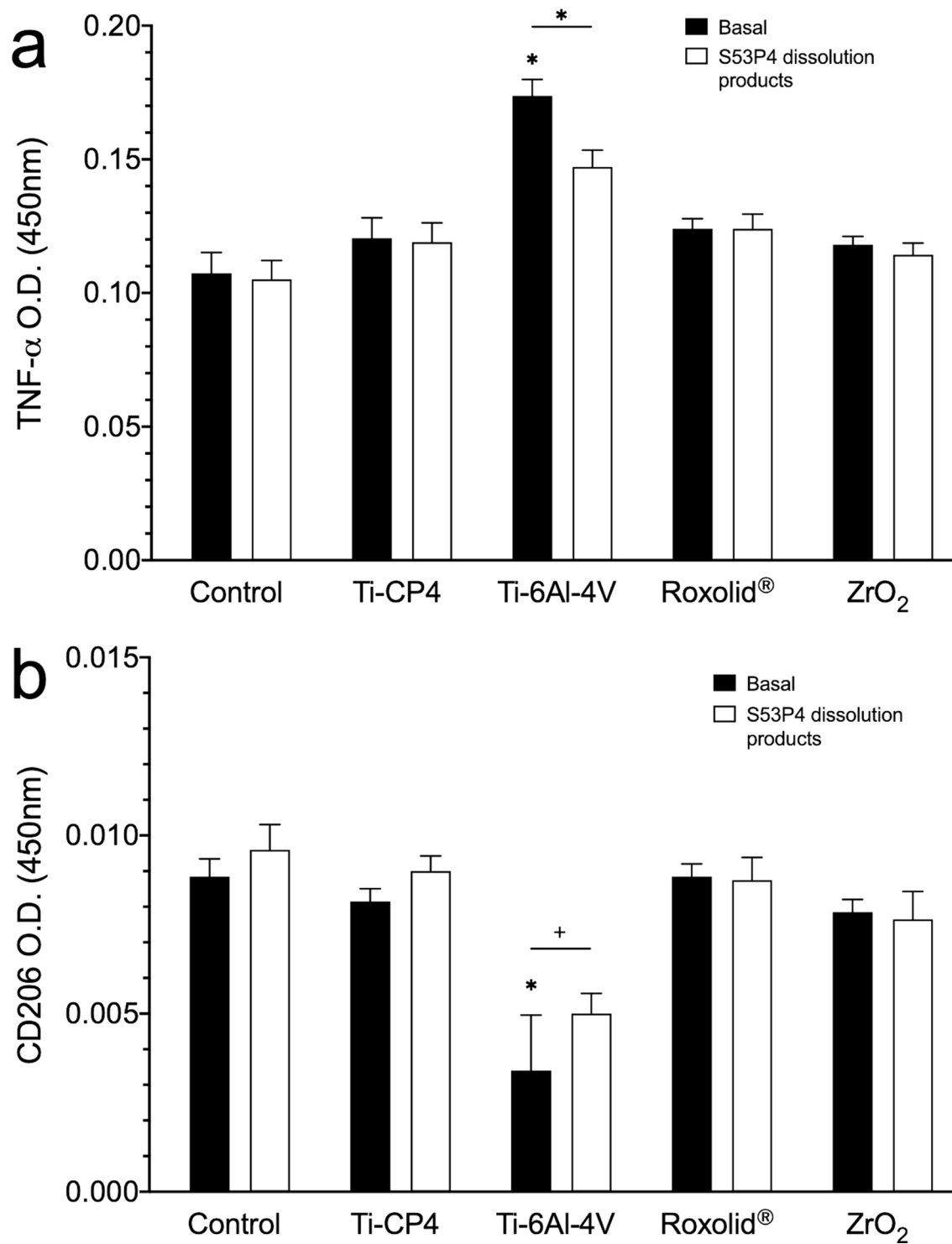


Fig. 3. Expression of typical macrophage polarisation markers. THP-1 derived macrophages were cultured in the presence of Ti-CP4 (commercially pure titanium), Ti-6Al-4V (grade 5 titanium alloy), Ti-15Zr (titanium-zirconium alloy) or ZrO₂ (zirconia) particles with or without S53P4 dissolution products for 24 h. Secretion of typical M1 macrophage marker TNF-α (a) and M2 macrophage marker CD206 (b) were measured using ELISA. * $p < 0.05$ and + $0.05 < p < 0.1$.

and IL-6 in several clinically relevant cell populations including human gingival fibroblasts, osteoblasts and bone marrow stromal cells (Fig. 1). The presence of dissolution products of S53P4 BGs (contained approx. 327.30 ± 2.01 ppm Ca, 51.34 ± 0.41 ppm P and 61.48 ± 1.17 ppm Si, pH 8.01 ± 0.21) suppressed the up-regulation of such pro-inflammatory markers induced by Ti-6Al-4V implant particles. While authors acknowledge a small set of inflammatory cytokines were investigated in this study, the results provide evidence to suggest that S53P4 BG may

have anti-inflammatory capabilities.

Similarly, the addition of S53P4 dissolution products suppressed the up-regulation of TNF-α and down-regulation of CD206 in THP-1 derived macrophages in the presence of Ti-6Al-4V particles (Fig. 3). TNF-α is a marker for M1 macrophages, which play a key role in the inflammatory response to foreign objects such as wear particles; CD206 is a marker for M2 macrophages, which play an important role in extracellular matrix remodelling [18,19]. Therefore a reduction in M1 and a higher number

of M2, relative to basal conditions indicates the dissolution products of S53P4 BG can play a role in polarisation of THP-1 derived macrophages towards M2 phenotype. the glass could potentially reduce inflammation due to particles lost by dental implants. This could in turn benefit the angiogenesis and extracellular matrix formation by endothelial cells and dermal fibroblasts if the glass was to be used in wound healing applications [15,20].

The mechanism behind this macrophage polarisation is likely to be due to silicate ions, in the dissolution products of S53P4 BG, modulating the expression of inflammatory and macrophage polarisation markers. A previous study reported that pro-inflammatory markers TNF- α and IL-6 in LPS (lipopolysaccharide) stimulated macrophages were lower in the presence of silicate based glasses in comparison to zinc phosphate glasses [11], suggesting soluble silica species might reduce inflammation and silica-based glasses be of clinical use in conditions associated with inflammation. There are also studies reported that Si ions at a certain concentration is highly beneficial in stimulating cell proliferation and osteogenic differentiation in osteoblasts [21,22]. However, further studies are needed to elucidate the mechanism through which BG and its ionic products act on cellular inflammatory response and macrophage behaviours. We also acknowledge that the ionic concentration reached a maximum value following 72 h of dissolution in culture medium *in vitro*, without any change of media. BG ion release profile may differ *in vivo*.

The use of BGs and subsequent integration of tissue engineering concepts in dentistry is an emerging field of applied research. Various trials and applications including dental adhesives, hypersensitivity, enamel remineralisation, restorative material, pulp capping and root canal therapy, bone substitutes in maxillofacial surgery and more have been reported [23–28]. There are already several products based on the original 45S5 BG. PerioGlas[®], the first bioactive glass particulate bone graft, has been used since 1996 for apical bone regeneration as well as a haemostatic agent for trabecular bone haemorrhage [1,29].

Titanium and titanium-based alloys are most widely used materials for dental implants. Although titanium-based materials are highly biocompatible and osteoconductive, they provide undesirable attachment sites for micro-organisms and are known to release wear particles, which have been shown to induce cytotoxicity as well as inflammation, resulting in peri-implantitis [8,30,31]. BGs are known for their ability to bond actively to bone, anti-microbial and as demonstrated in the current study, potential anti-inflammatory capabilities. We believe the addition of BG can potentially benefit traditional dental implants. One of the challenges in incorporating BG with dental implants is the thermal expansion coefficient. As glass and metal shrink at different rates during cooling, BGs can be prone to cracks [32]. Adjustments to BG thermal expansion coefficient can be achieved by alterations to glass composition such as silica content or substitution of calcium and/or sodium [33]. Various methods have been investigated for stable incorporation of BGs with dental implants, these include enamelling, sol-gel, electrophoretic deposition, laser cladding, thermal spray and radio-frequency magnetron sputtering [23,32]. While some studies have demonstrated promising results such as improved osseointegration [34,35], currently there is no commercially available BG incorporated dental implants available for clinical use, this emphasises the need of further efforts in the development of new generation of BG incorporated dental implants.

Conclusion

We have proven the hypothesis that S53P4 BG possesses anti-inflammatory properties to peri-implant cell populations such as gingival fibroblasts, osteoblasts and bone marrow derived stromal cells and, potentially affect macrophage activities through the release of dissolution ions. Further studies are required to fully understand the reliability, efficiency and exact mechanisms of action in the search for new generation of treatment modalities in dentistry.

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CRediT authorship contribution statement

Fadi N. Barrak: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Siwei Li:** Conceptualization, Methodology, Investigation, Data curation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ali A. Mohammed:** Investigation, Data curation, Supervision, Writing – review & editing. **Connor Myant:** Funding acquisition. **Julian R. Jones:** Supervision, Writing – review & editing.

Conflicts of interest

The 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.

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