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BIOCOMPATIBILITY STUDIES

Original Research

Unraveling the immunomodulatory and metabolic effects of bioactive glass S53P4 on macrophages in vitro

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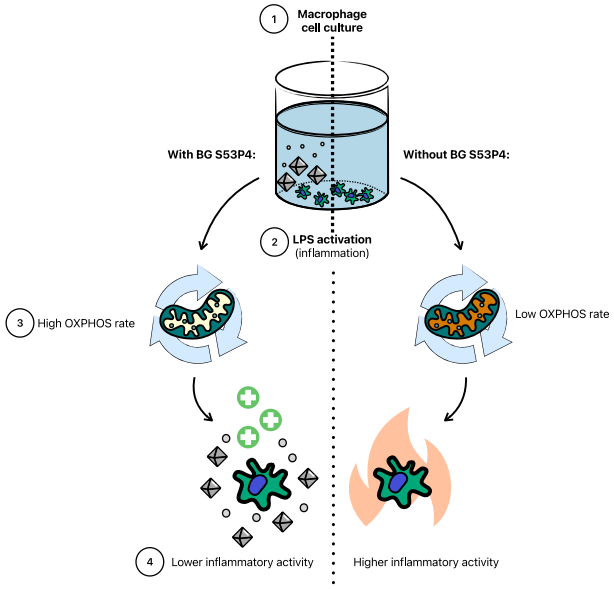
Abstract

Macrophage metabolism is closely linked to their phenotype and function, which is why there is growing interest in studying the metabolic reprogramming of macrophages. Bioactive glass (BG) S53P4 is a bioactive material used especially in bone applications. Additionally, BG S53P4 has been shown to affect macrophages, but the mechanisms through which the possible immunomodulatory effects are conveyed remain unclear. According to the results presented here, the lipopolysaccharide (LPS) induced suppression in oxidative phosphorylation is rescued in macrophages cultured with BG S53P4 before the inflammatory stimulus. Additionally, BG S53P4-exposed macrophages expressed lower mRNA levels of inflammatory cytokines *Il6* and *Il1b*, as well as demonstrated decreased activation of inflammatory interferon regulatory factor (IRF) and NF-κB pathways and nitrogen oxide secretion in response to LPS. These results did not rely on cells being in direct contact with the material as similar effects were observed in the presence of BG S53P4-conditioned medium. Our findings link the immunomodulatory properties of BG S53P4 and macrophage metabolism, which improves our understanding of the mechanisms underlying the clinical efficacy of bioactive glasses.

Graphical Abstract



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1 Introduction

Macrophages are a versatile immune cell type and their activation has proven to be crucial for inflammatory processes, including the resolution of inflammation. Stimulation of macrophages by the toll-like receptor ligand lipopolysaccharide (LPS) leads to activation of the transcription factor nuclear factor-kappa B (NF-κB), which in turn triggers a wide range of cellular responses. These cells produce inflammatory cytokines and chemokines, such as tumor necrosis factor (TNF), interleukin 1 beta (IL1β), and IL6 [1], which are often used to classify the macrophages as inflammatory. During healing processes, macrophages shift from inflammation promoting to anti-inflammatory cells in order to advance the resolution of inflammation and thereby tissue repair [2]. This essential conversion, occurring during processes such as wound healing or bone tissue repair [2, 3], involves macrophages that express markers like transforming growth factor beta (TGFβ) and mannose receptor C-type 1 (MRC1)/CD206, which are commonly used to classify them as anti-inflammatory [4].

In addition to inflammatory responses, inflammatory and anti-inflammatory macrophages have been shown to display distinct metabolic profiles. Generally, inflammatory macrophages prefer glycolytic metabolism, while anti-inflammatory macrophages use oxidative phosphorylation (OXPHOS) [4]. Since macrophage metabolism is closely linked to their phenotype and function, there is a growing interest in the metabolic reprogramming of macrophages, which could even be utilized as a novel therapeutic approach.

Regarding macrophage metabolism, two competing arginine pathways function at the center of macrophage polarization [5]. In inflammatory macrophages, the inducible nitric oxide synthase (iNOS or NOS2) metabolizes arginine into nitric oxide (NO) that can be further metabolized into reactive nitrogen species. By contrast, in anti-inflammatory macrophages, the active arginase (ARG) enzyme hydrolyses arginine to urea and ornithine, the latter eventually being important for cellular proliferation and tissue repair [5].

Bioactive glasses were discovered in the 1970s [6] and since then, different compositions have been developed to be used as bone fillers and implants. A silica-based bioactive glass (BG) S53P4 is composed of silicon dioxide, sodium oxide, calcium oxide, and phosphorous pentoxide [7]. BG S53P4 is known to release sodium and calcium ions, and silicon species into its environment, and to increase the pH of surrounding solution, which are linked to the osteogenic effects caused by the BG [8].

In clinical setting, BG S53P4 has been proven to be effective in the treatment of osteomyelitis, which is an inflammatory and infectious process in the bone [9]. In preclinical studies, BG S53P4 has been shown to possess antimicrobial properties against a variety of clinically important aerobic pathogens [10] and oral microorganisms [11]. These antimicrobial effects are suggested to be due to change in pH, osmotic effects, and Ca^{2+} concentration [12]. In recent years, there has been a growing interest in the immunomodulatory functions of bioactive glasses. For example, BG S53P4 has been shown to reduce the levels of inflammatory cytokines, such as TNF and IL1β in LPS-challenged macrophages [13] as well as the amount of secreted TNF in the presence of titanium alloy particles [14].

Even though there are studies on bioactive glasses and macrophages, the mechanisms through which BGs exert their immunomodulatory effects still remain unclear. So far, most studies have addressed the activation status and polarization of macrophages in response to bioactive glasses without considering e.g., the potential effects on metabolism. Therefore, the main aim of the current study was to explore the responses of LPS-activated macrophages to BG S53P4. The potential metabolic rewiring was studied by assessing mitochondrial respiration and glycolytic rate, while the phenotype and function were studied by examining the differentially expressed genes and inflammatory signaling pathways, as well as NO production in macrophages challenged with BG S53P4. With this approach, we aimed to provide a more comprehensive understanding of the effects of bioactive glasses on macrophage phenotype and function.

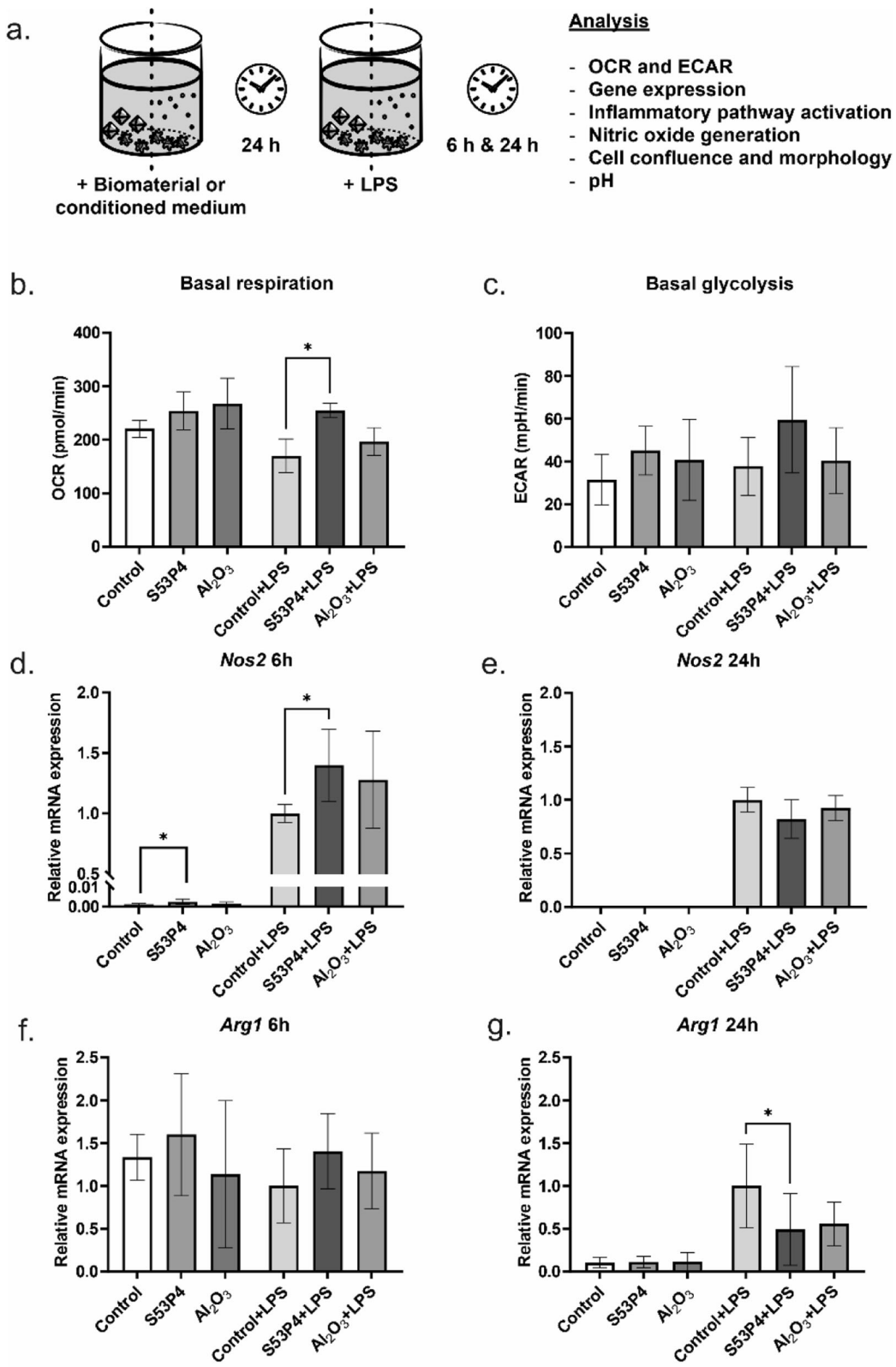
2 Materials and methods

2.1 Biomaterials

Bioactive glass S53P4 (23% Na_2O, 53% SiO_2, 4% P_2O_5, and 20% CaO; manufacturer: Mo-SCI Health Care L.L.C. 4040 HyPoint North, Rolla MO 65401 USA) with a particle size of 500–800 µm was used in this study. As a control material, we used biologically inert Al_2O_3 (99.75% Al_2O_3, 0.25% Na_2O, 0.02% SiO_2, 0.02% Fe_2O_3; manufacturer: Duralum White, Washington mills USA) with a particle size of 500 µm. Before using in the experiments, biomaterials were sterilized by dry heat (180 °C, 4 h).

2.2 Murine cell line cultures and study design

Murine-derived RAW 264.7 (ATCC, TIB-71) and J774-Dual (InvivoGen, j774d-nfis) macrophage-like cells were cultured in phenol-red containing Dulbecco's modified Eagle's medium (Gibco, 41966-029) unless otherwise



stated. All cell cultures were supplemented with heat-inactivated fetal bovine serum (10%, Gibco, 10270-106) and Penicillin-Streptomycin (100 U/ml-100 µg/ml, Gibco, 15140-148). The J774-Dual cells medium was further supplemented with Normocin (100 µg/ml, InvivoGen, ant-nr-1) and selective antibiotics Blasticidin (5 µg/ml,

◀ **Fig. 1** Illustration of the experimental design (**a**) Macrophages were cultured in contact with bioactive glass (BG) S53P4 or Al_2O_3, or in biomaterial conditioned medium for 24 h and then activated with LPS. Analyses were conducted at 6 h (for gene expression) and 24 h (for all) after LPS activation. Mitochondrial oxidative phosphorylation and glycolytic rate of RAW 264.7 macrophages as evaluated by the oxygen consumption rate (OCR) (**b**) and extracellular acidification rate (ECAR) (**c**) in cells cultured in BG S53P4 or Al_2O_3 conditioned media for 48 h. Expression of genes related to macrophage metabolism. i.e., *Nos2* (**d**, **e**) and *Arg1* (**f**, **g**) in cells cultured in contact with BG S53P4 or Al_2O_3 after 6 and 24 h of LPS induction. The gene expression data is normalized to LPS-activated control group. All data are presented as the mean ± SD of three independent biological experiments with three technical replicates per each experimental condition. $p^* \leq 0.05$ (ANOVA followed by Dunnett's multiple comparisons test). *LPS* lipopolysaccharide

Invivogen, ant-bl-1) and Zeocin (100 µg/ml, Invivogen, ant-zn-1). The cells were maintained at +37 °C, 5% CO_2, unless otherwise stated. The growth media for RAW 264.7 cells was changed every 2 to 3 days, and the cells were passaged before reaching confluency. J774-Dual cells were passaged every three days. J774-Dual cells at passages 8–10 and RAW 264.7 cells at passages 13-16 were used for experiments. All cell culture experiments followed the same experimental design (Fig. 1a): macrophages were first cultured in contact with the biomaterials or in biomaterial-conditioned media for 24 h, after which they were activated with bacterial lipopolysaccharide (LPS, 10 ng/ml, Invitrogen, 00-4976-93) for 24 h, except for gene expression analysis by qPCR, for which also a time point of 6 h were included. The total culture time before analysis was thus 48 h in most experiments, unless otherwise stated.

2.3 Preparation of material conditioned media

To obtain dissolution products of biomaterials, conditioned media was prepared by adding bioactive glass S53P4 or Al_2O_3 in a concentration of 15 mg per ml of growth media into T25 cell culture flasks. The flasks were placed in cell culture incubator (+37 °C, 5% CO_2) and the media were conditioned for 48 h, after which the media were collected and centrifuged before they were used in experiments. Similarly, growth medium without biomaterials was incubated for 48 h and used as a control medium. For every experiment, new conditioned media were prepared, and, in each experiment, the cell culture medium was replaced with full volume (100%) of conditioned medium.

2.4 Measurement of oxygen consumption and extracellular acidification rates

The Agilent Seahorse XFe96 analyzer (Agilent, North Billerica, MA, USA) was used to measure the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of live RAW 264.7 cells. As bioactive glass S53P4 is known to increase the pH of surrounding medium, the experiment was performed both in normal cell culture conditions (+37 °C, 5% CO_2) and with a lower level of CO_2 (3.8%). The lower CO_2 level alters the buffering capacity of sodium bicarbonate buffer in the medium, resulting in a higher pH level of 7.8. This adjustment allowed us to study if the responses observed in macrophages cultured in the presence BG S53P4 are dependent on the increased pH level associated with the bioactive glass.

The cells were seeded in Seahorse XFe96 cell culture microplates (94,000 cells/cm^2) in 80 µl of BG S53P4 or Al_2O_3 conditioned media following the manufacturer's instructions. For each experimental condition, 14–16 technical replicates were included. After 24 h LPS (10 ng/ml) was added in half of the wells. After another 24 h of LPS activation, the medium was changed into HEPES-buffered Seahorse XF DMEM with pH of 7.4 (Agilent, 103575-100) supplemented with 2 mM glutamine (Agilent, 103579-100), 1 mM sodium pyruvate (Agilent, 103578-100) and 10 mM glucose (Agilent, 103577-100). The cells were kept in CO_2-free incubator at 37 °C for 1 h. After this, the OCR and the ECAR were measured according to the manufacturer's instructions. The whole experiment was repeated three times, both in normal cell culture conditions (+37 °C, 5% CO_2) and with a lower, 3.8%, CO_2 level.

2.5 RNA isolation and quantitative PCR

RAW 264.7 cells were seeded in a 6-well culture plates (50,000 cells/cm^2, in 3 ml of medium). The cells were cultured either in direct contact with bioactive glass S53P4 or Al_2O_3 (45 mg each, 15 mg/ml). Cells cultured without biomaterials served as controls. Three technical replicates were included for each condition. After 24 h, half of the cultures were supplemented with LPS (10 ng/ml). After 6 and 24 h from the LPS activation, the cells were lysed and total RNA was extracted with NucleoSpin RNA Plus, Mini Kit (Macherey-Nagel, 740984) following the manufacturer's instructions. After determining the RNA concentrations using NanoDrop One spectrophotometer (Thermo-Scientific), the RNA was reverse-transcribed into cDNA using High-Capacity cDNA Reverse Trancription Kit (Applied Biosystems, 5368814), and 20 ng of cDNA was used for amplification of target genes with qPCR using the Taqman® gene expression assays (Applied Biosystems). The genes of interest and Taqman® assay IDs are listed in Supplementary Table S1. Actin beta was used as a reference gene. Gene expression was quantified using the ΔΔCt method and fold change was calculated with the $2^{-\Delta\Delta Ct}$ formula. The whole experiment was repeated as three biological replicates.

2.6 Evaluation of NF-κB and IRF pathway activity in J774-Dual cells

J774-Dual cells were seeded in 24-well culture plates (11,000 cells/cm^2, in 1 ml of medium) for 24 h either in the presence of bioactive glass S53P4 or Al_2O_3 (15 mg/ml each) or in biomaterial conditioned media. Cells cultured without biomaterials served as control. Three technical replicates were included for each experimental condition. Growth medium without cells was also incubated in the same plate to serve as an assay blank. After 24 h, LPS (10 ng/ml) was added to half of the wells. After another 24 h, medium samples of 700 µl were collected from each well.

Quanti-Blue (InvivoGen, rep-qbs) was used for the detection and quantification of secreted embryonic alkaline phosphatase (SEAP), allowing the investigation of NF-κB pathway. Quanti-Blue medium was prepared according to manufacturer's instructions and 180 µl per well was added in the wells of a 96-well plate, followed by addition of 20 µl of medium sample collected from cells (see above) per well in triplicates. After 4-h incubation, the absorbance was measured at 650 nm using EnSight™ Multimode plate reader (PerkinElmer).

Quanti-Luc (InvivoGen, rep-qlc4g2) was used to detect luciferase reporter enzyme corresponding to interferon regulatory factor (IRF) pathway activation. Medium samples collected from cells (see above) were first pipetted on a 96-well plate at 20 µl per well in triplicates. Thereafter Quanti-Luc medium was prepared according to manufacturer's instructions, 50 µl per well was added and the luminescence was immediately measured using EnSight™ Multimode plate reader (PerkinElmer). The whole experiment was repeated as three biological replicates.

2.7 Measurement of nitrite (NO_2^-) generation

Griess reagent (modified, Sigma-Aldrich, G4410) was used to measure the levels of nitrite in cell culture supernatants. For this colorimetric assay, phenol red-free DMEM (Gibco, 31053-028) was used and further supplemented with L-glutamine (3.9 mM, Agilent, 103579-100) to be equal to the L-glutamine concentration of the phenol red-containing DMEM used in other experiments.

RAW 264.7 macrophages were seeded in 24-well plates (94,000 cells/cm^2, in 1 ml of medium) for 24 h both together with bioactive glass S53P4 or Al_2O_3 (15 mg/ml each), or in biomaterial conditioned media in triplicates. Three technical replicates were included for each experimental condition. After 24 h, LPS (10 ng/ml) was added to half of the wells. After another 24 h, medium samples were collected from each well for the measurement of nitrite by Griess reagent according to the manufacturer's instructions. Shortly, 50 µl of culture supernatant with 50 µl of Griess reagent was pipetted on a 96-well plate in triplicates and absorbance at 540 nm was read after 15 min. Serial dilutions (0–100 µM) of sodium nitrate stock solution (10 mM in ddH_2O) were prepared in cell culture medium to obtain a standard curve. The whole experiment, including samples from cells cultured both with biomaterials and in conditioned media, was repeated as three biological replicates.

2.8 Measuring cell confluence with IncuCyte real-time live cell imaging

The IncuCyte Live-Cell Analysis System (Sartorius) was used to assess growth rate and cell confluence. For this, RAW 264.7 macrophages were seeded in 24-well plates (18,500 cells/cm^2, in 1 ml of medium) both together with bioactive glass S53P4 or Al_2O_3 (15 mg each), or in biomaterial conditioned media in triplicates. After 24 h, LPS (10 ng/ml) was added to half of the wells. The cells were automatically imaged real-time by the IncuCyte system every hour up to 48 h with a 10× objective. Cell confluence was automatically calculated by the IncuCyte Basic Analysis Software in a single representative experiment and macrophage morphology, i.e., cell size, shape, and granularity, was visually evaluated from under images obtained with IncuCyte.

2.9 Measuring the pH of biomaterial conditioned media

To measure the pH-change in culture medium caused by BG, conditioned media was prepared as described above. After 48 h, the flasks were removed from the cell culture incubator and the pH in the medium was immediately measured using a standard pH meter (Mettler Toledo). The measurements were repeated three times as individual experiments.

2.10 Measuring pH in cell cultures

To measure the pH in cell culture, a fiber optic pH sensor dish reader (PreSens Precision Sensing GmBh) was applied. RAW 264.7 cells (94,000 cells/cm^2, in 1 m of medium) were seeded in a PreSens 24-well HydroDish plate, with pH sensors integrated at the bottom of each well. The cells were cultured in the presence of bioactive glass S53P4 or Al_2O_3 (15 mg/ml each), or in biomaterial conditioned media. After 24 h, LPS (10 ng/ml) was added into half of the wells, after which pH in each well was automatically recorded every hour up to 48 h. Two technical replicates were included for each experimental condition and the whole experiment was performed once.

2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA). The data from non-activated and LPS-activated cells were tested separately, and comparisons were done between the experimental group of interest and the corresponding control group. Multiple comparisons for normally distributed data were done using ordinary one-way ANOVA, followed by Dunnett's multiple comparisons test. Kruskal-Wallis test followed by Dunn's multiple comparisons test were performed on non-normally distributed data. All reported p-values are multiplicity adjusted. Differences with p-values equal to or less than 0.05 were considered statistically significant, and levels of significance being indicated as follows: *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$, p****< 0.0001. All values are presented as mean ± standard deviation, unless otherwise stated.

3 Results

3.1 BG S53P4 prevents LPS-induced OCR suppression and modulates the transcription of *Nos2* and *Arg1*

To assess how BG S53P4 affects macrophage metabolism, mitochondrial respiration and glycolytic rate in RAW 264.7 cells were evaluated by measuring the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) using the Agilent Seahorse XFe96 analyzer. Generally, LPS activation decreased the basal respiration rate (OCR, Fig. 1b). However, when macrophages were cultured in BG S53P4 conditioned medium before LPS activation, OCR remained higher compared to LPS-activated control ($p = 0.01$, Fig. 1b). A slightly higher ECAR, indicating increased basal glycolysis, was measured from cells cultured in the presence of BG S53P4 conditioned medium, although this trend did not reach statistically significant difference (Fig. 1c). The observed increase in OCR and ECAR however suggest that BG S53P4 can induce a global change in energy demand of LPS-activated cells.

The expression of inducible nitric oxide synthase (*Nos2*) and arginase (*Arg1*) genes, which are related to macrophage metabolism, were evaluated in RAW 264.7 macrophages using qPCR after 6 and 24 h of LPS activation. As expected, *Nos2* mRNA was upregulated in LPS-activated macrophages (Fig. 1d, e). Interestingly, at an earlier 6-h time point, which was specifically included in gene expression analysis, macrophages cultured in contact with BG S53P4 expressed higher *Nos2* mRNA levels compared to control cells without LPS ($p = 0.023$, Fig. 1d). Similarly, cells treated with BG S53P4, and LPS-treated cells expressed higher *Nos2* compared to only LPS-activated control cells ($p = 0.017$, Fig. 1d). However, after 24 h, there were no longer significant differences between cells cultured in contact with BG S53P4 or control cells, with or without LPS-activation (Fig. 1e).

After 6 h, no statistically significant differences in mRNA levels of *Arg1* were detected (Fig. 1f), but after 24 h, *Arg1* mRNA levels were high in cells treated with LPS, while low levels were detected from cells without LPS-activation (Fig. 1g). In this later time point, macrophages cultured in direct contact with BG S53P4 before LPS-activation displayed a lower level of *Arg1* mRNA expression compared to LPS-activated control cells ($p = 0.031$, Fig. 1g).

Taken together, BG S53P4 transiently increases the transcription of the *Nos2*, which in inflammatory macrophages encodes the inducible nitric oxide synthase that metabolizes arginine into nitric oxide [5]. Furthermore, BG S53P4 reduced the transcription of *Arg1* in LPS-activated macrophages, which is commonly related to healing promoting functions [5]. Based on these results, it seems that BG S53P4 could actually skew macrophage arginine metabolism towards a more inflammatory direction during the first days of cell culture.

The chemically inert Al_2O_3 had no significant effects on RAW 264.7 macrophage mitochondrial respiration or glycolytic rate, or on the expression levels of the two metabolism related genes, *Nos2* and *Arg1*, which were investigated in this setting (Fig. 1d–g).

3.2 BG S53P4 reduces the inflammatory pathway activity and NO production in LPS-activated macrophages

Macrophage function in response to BG S53P4 and Al_2O_3 was studied by assessing the activity of interferon regulatory factor (IRF) and NF-κB pathways in J774-Dual macrophages. As expected, LPS increased the activity of both of these inflammation related pathways (Fig. 2a–d). Importantly, in macrophages cultured in direct contact with BG S53P4 before LPS activation, a significantly lower activity of both IRF ($p \le 0.001$, Fig. 2a) and NF-κB ($p = 0.001$, Fig. 2c) pathways was observed. Similar results were obtained when macrophages were cultured in BG S53P4 conditioned medium prior to LPS-activation; the activity of both IRF ($p = 0.038$, Fig. 2b) and NF-κB ($p < 0.0001$, Fig. 2d) pathways remained lower compared to LPS-activated control cells. Notably, the activity of NF-κB pathway was also lower when macrophages were cultured in direct contact with Al_2O_3 ($p = 0.041$, Fig. 2c) or in Al_2O_3 conditioned medium ($p = 0.004$, Fig. 2d) prior to LPS-activation.

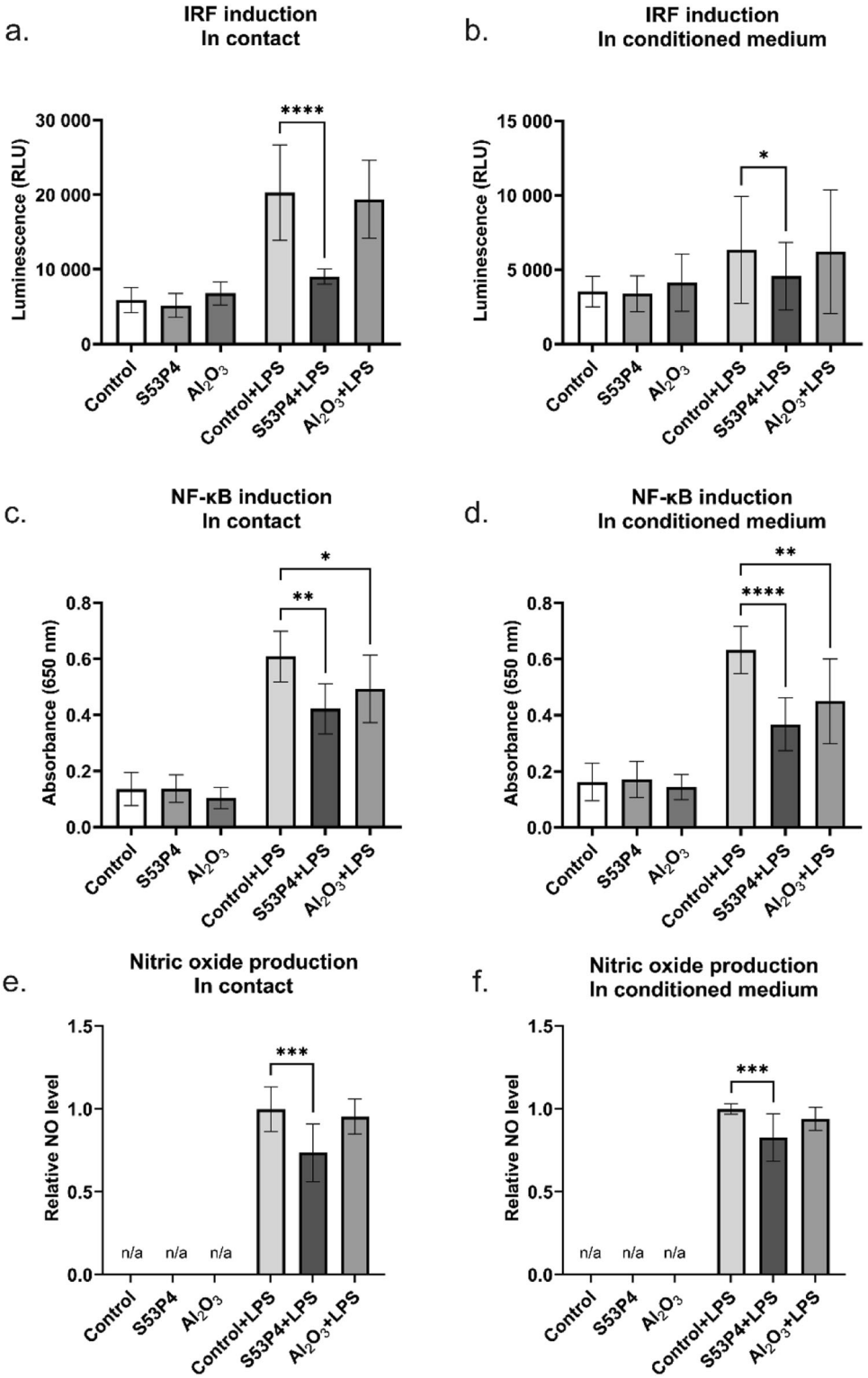


Fig. 2 The activity of interferon regulatory factor (IRF) (**a**, **b**) and NF-κB (**c**, **d**) pathways in J774-Dual macrophages in response to bioactive glass (BG) S53P4 and Al_2O_3 both in contact with the biomaterials or in biomaterial-conditioned media. Nitrite oxide (NO) secretion from RAW 264.7 macrophages after culturing cells in contact with BG S53P4 or Al_2O_3 (**e**) or with biomaterial conditioned media (**f**) for 48 h measured as nitrite using Griess reagent. NO production results are presented as nitrite levels measured as absorbance and then normalized to the corresponding LPS-activated control. All data are presented as the mean ± SD of three independent experiments with three technical replicates per group. $p^* \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$, $p^{****} < 0.0001$ (ANOVA followed by Dunnett's multiple comparisons test or Kruskal-Wallis followed by Dunn's multiple comparisons test for **a** and **f**). LPS lipopolysaccharide, n/a not applicable

Since nitric oxide (NO) produced by macrophages is recognized as an important signaling molecule in macrophage immunometabolism [15], we investigated if BG S53P4 affects the NO secretion from RAW 264.7 macrophages under LPS-stimulus. This was done by measuring nitrite levels in cell culture, which correspond to NO production. As expected, the cells secreted NO in response to LPS, while the levels remained under the assay detection level (10 nM) in the absence of LPS (Fig. 2e, f). Compared to LPS-activated control cells, NO levels

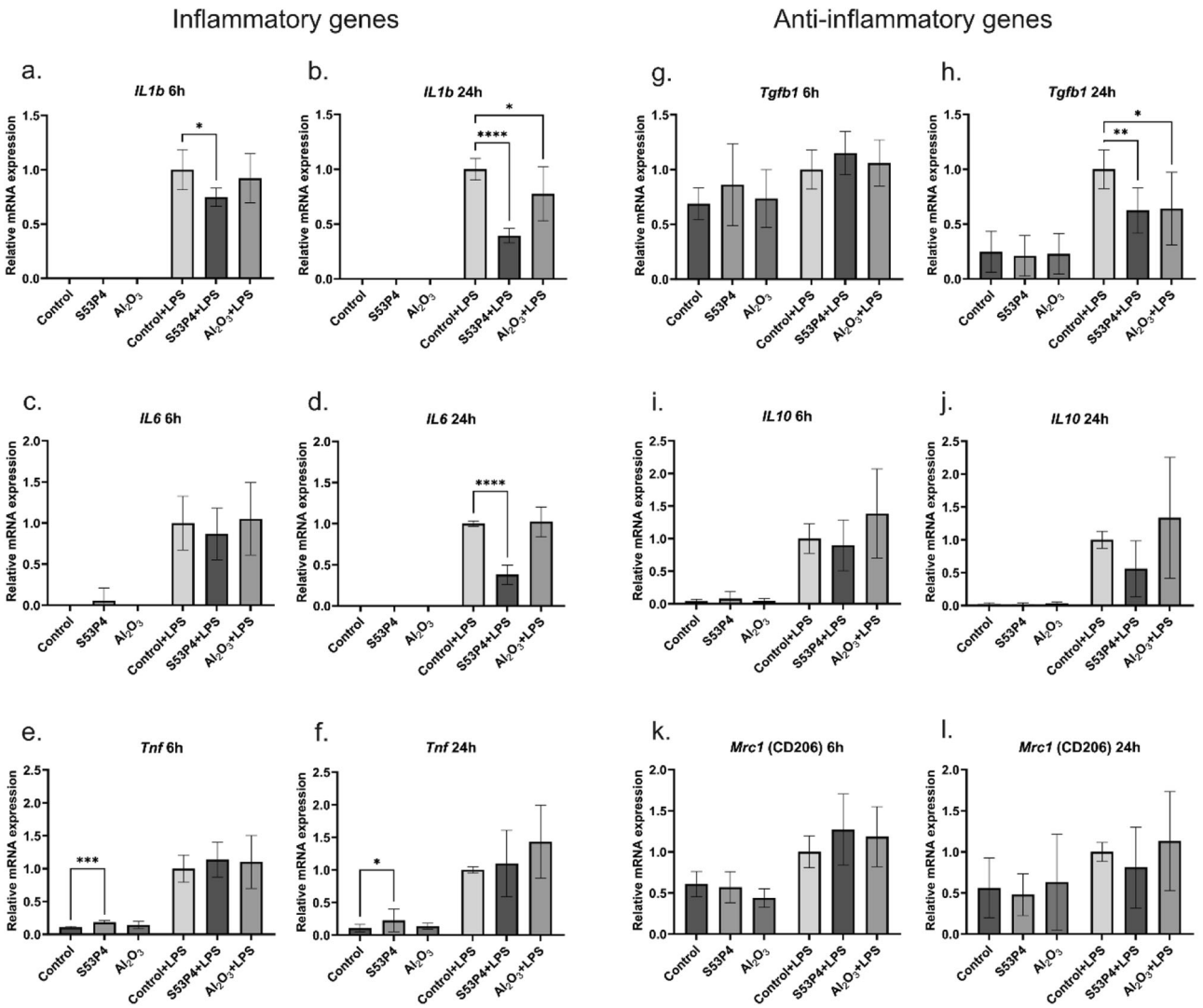


Fig. 3 Expression of inflammatory (**a**–**f**) and anti-inflammatory (**g**–**l**) genes related to macrophage function and activity. The mRNA expression levels were determined by qPCR in RAW 264.7 macrophages grown in contact with the bioactive glass (BG) S53P4 and Al_2O_3 with or without LPS induction after 6 and 24 h of culture. Results are normalized to the LPS-activated control group and all data are presented as the mean ± SD from three independent experiments with three technical replicates per group. p* ≤ 0.05, p** ≤ 0.01, p*** ≤ 0.001, p**** < 0.0001 (ANOVA followed by Dunnett's multiple comparisons or Kruskal-Wallis followed by Dunn's multiple comparisons test for **f**). LPS lipopolysaccharide

were significantly lower when macrophages were cultured in direct contact with BG S53P4 ($p < 0.001$, Fig. 2e) or in BG S53P4 conditioned medium ($p < 0.001$, Fig. 2f) before LPS-activation, while Al2O3 had no effect on NO production in either experimental condition (Fig. 2e, f).

3.3 Culturing macrophages with BG S53P4 alters *Il1b*, *Il6* and *Tgfb1* mRNA expression after LPS activation

To further analyze macrophage function, the expression levels of certain inflammatory and anti-inflammatory genes were examined by qPCR in cells cultured in direct contact with BG S53P4 or Al_2O_3. As expected, LPS activation induced the transcription of inflammatory genes *Il1b*, *Il6* and *Tnf* (Fig. 3a–f). Importantly, macrophages, which had been in contact with BG S53P4 before LPS activation showed lower mRNA expression levels of inflammatory cytokines *Il1b* (at 6 h: $p = 0.015$, Fig. 3a; at 24 h: $p < 0.0001$, Fig. 3b) and *Il6* (24 h: $p < 0.0001$, Fig. 3d) compared to LPS-activated control cells. Similarly, in macrophages cultured in contact with Al_2O_3 before LPS activation, the expression of *Il1b* mRNA remained lower at 24 h ($p = 0.022$, Fig. 3b).

Interestingly, even without LPS activation, the mRNA expression level of *Tnf* was significantly higher in macrophages cultured in contact with BG S53P4 when compared to control cells (at 6 h: $p = 0.001$, Fig. 3e; at 24 h: $p = 0.012$, Fig. 3f). However, after LPS activation, no

difference in *Tnf* mRNA expression was observed between cells cultured in contact with BG S53P4 or Al_2O_3, or LPS-activated control cells (Fig. 3e, f).

Of the anti-inflammatory genes, *Tgfb1* mRNA expression was the most substantially affected by BG S53P4. Initially, no significant differences in *Tgfb1* mRNA expression were observed at 6 h (Fig. 3g). However, at 24 h, the expression of *Tgfb1* mRNA was significantly lower in macrophages that were cultured in contact with BG S53P4 before LPS-activation ($p = 0.009$, Fig. 3h). Culturing macrophages in contact with Al_2O_3 prior to LPS-activation had a similar effect, and *Tgfb1* mRNA expression was significantly lower compared to LPS-activated control cells ($p = 0.015$, Fig. 3h).

The mRNA levels of the anti-inflammatory *Il10* were clearly increased by LPS, as *Il10* mRNA was barely measurable from macrophages without LPS-treatment (Fig. 3i, j). LPS also seemed to increase the mRNA expression of mannose receptor C-type 1, *Mrc1* (CD206) compared to cells without LPS treatment (Fig. 3k, l). However, culturing macrophages in contact with either biomaterial BG S53P4 or Al_2O_3 before LPS activation did not significantly affect the mRNA expression of these genes.

Taken together, culturing macrophages in contact with BG S53P4 before LPS-activation notably reduces the mRNA expression of inflammatory interleukins *Il1b* and *Il6*, and of the anti-inflammatory *Tgfb1*.

3.4 Macrophages proliferate slower in contact with biomaterials but morphology remains unaffected

Macrophage growth rate and confluence was analyzed using the IncuCyte Basic Analysis Software. As expected, an overall increase in cell proliferation was observed after LPS activation (Fig. 4a, b). However, cell confluence at 48 h was lower when macrophages were cultured in direct contact with either BG S53P4 ($p = 0.002$) or Al_2O_3 ($p = 0.005$) when compared to control cells treated only with LPS (Fig. 4c). Conversely, when the cells were cultured in biomaterial conditioned medium, no such effect was observed, and cell confluence in cultures performed in the presence of BG S53P4 or Al_2O_3 and LPS were equal to LPS-activated control cultures (Fig. 4d). As the growth of cells was microscopically monitored during every experiment, the representative experiment with IncuCyte system was performed only once and thus analyzing replicates statistically within a single experiment does not provide reliable information regarding experimental reproducibility.

The effects of biomaterials and LPS on RAW 264.7 macrophage morphology were qualitatively assessed from phase contrast images obtained with the IncuCyte using 20× magnification (Fig. 4e). Non-activated control cells were rounded with an oval morphology, and typically exhibited only a few pseudopods. While nuclear borders were not clearly distinguishable, nucleoli were more recognizable. LPS-activated control cells were larger and had a more varied morphology compared to non-activated cells. They appeared swollen and several cells had many membrane protrusions extending from the cell body. The cytoplasm was clearly granular with numerous vesicles. No distinguishable morphological differences were observed between cells cultured either in contact with BG S53P4 or Al_2O_3, or in biomaterial conditioned media, and control cells (with or without LPS) (Fig. 4e).

3.5 The increase in pH caused by BG S53P4 could contribute to the metabolic rewiring in macrophages

As BG S53P4 is known to increase the pH of its surroundings [8], we investigated how the pH is affected in cell cultures by measuring pH in RAW 264.7 macrophage cultures with the PreSens sensor dish reader. The pH was approximately 0.2 units higher in cell cultures performed in direct contact with BG S53P4 (Fig. 5a) or in BG S53P4 conditioned medium (Fig. 5b). Over 48 h, the pH steadily decreased, probably as a result of macrophage metabolism and this decrease occurred both with and without LPS activation. Nevertheless, the pH remained higher throughout the experiment when compared to Al_2O_3-treated or control cell cultures (Fig. 5a, b). A similar increase in pH that was observed in cell cultures, was measured from cell culture medium conditioned with BG S53P4 after 48, and this increase was statistically significant compared to control or Al_2O_3 conditioned medium ($p = 0.001$ for both, Fig. 5c).

To further examine if the effects of BG S53P4 on macrophage metabolism are mediated by pH increase, we again evaluated the mitochondrial respiration of cells using the Agilent Seahorse XFe96 analyzer. For this, the CO_2 level inside the cell culture incubator was adjusted to 3.8%, since this alters the pH of bicarbonate buffered cell culture medium to 7.8, and the RAW 264.7 macrophage cell cultures were kept in this pH prior to the assay. Unlike in previous experiments, which were completed in pH 7.4 (Fig. 1b, c), no increase in either OCR (Fig. 5d) or ECAR (Fig. 5e) were detected in macrophages cultured in BG S53P4 conditioned medium. This suggests that the effects of BG S53P4 on macrophage metabolism described in this study could be, at least partially, mediated by the increased pH resulting from the BG dissolution in cell culture medium.

4 Discussion

Bioactive glass (BG) S53P4 has been shown to possess antimicrobial properties and therefore shows promise in

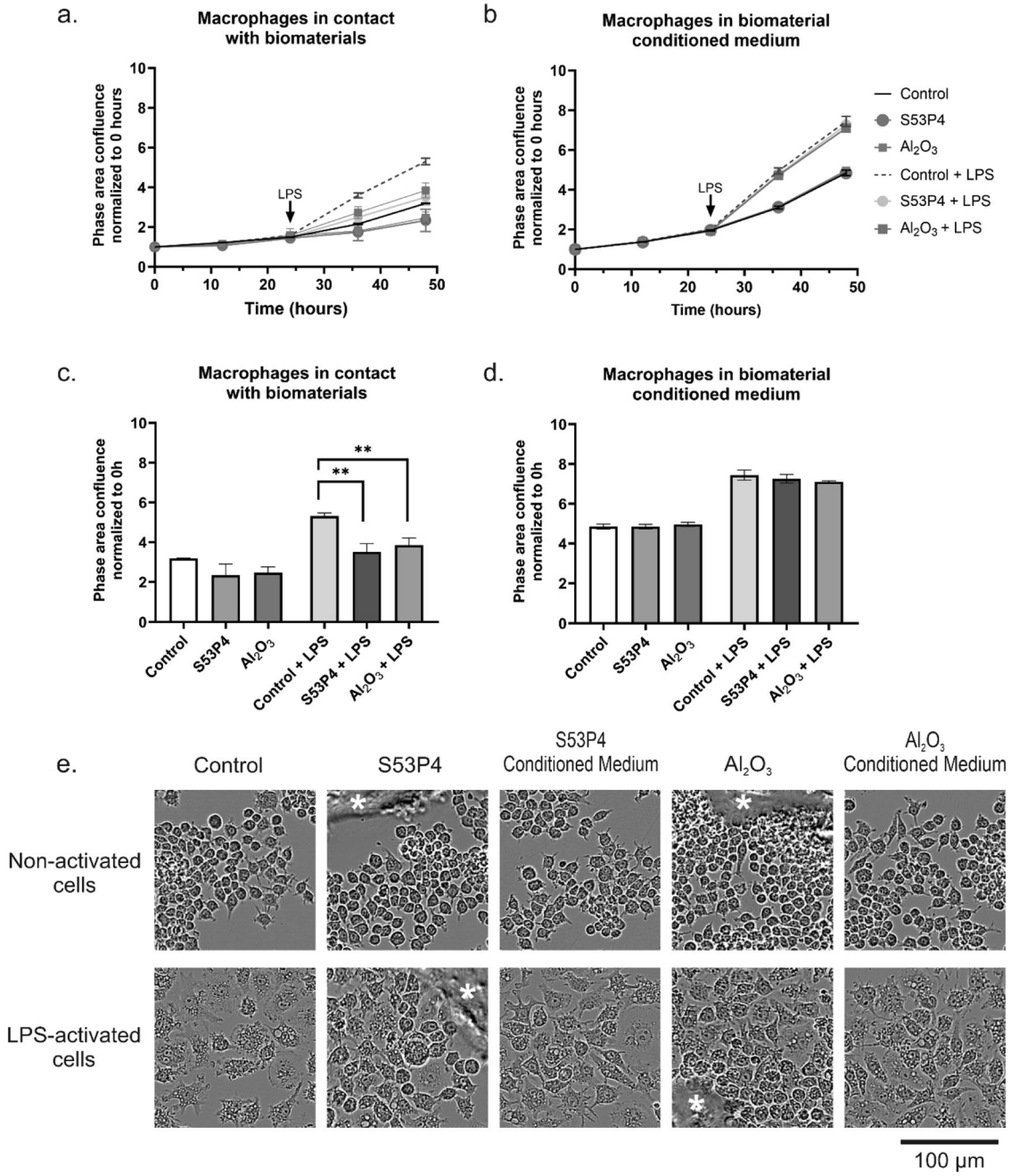


Fig. 4 Confluency of RAW 264.7 macrophage cultures reflecting cell growth rate was analyzed with the IncuCyte real-time cell imaging system up to 48 h (**a**, **b**). Bar graphs and statistical comparisons of macrophage confluency at the end point of 48 h (**c**, **d**). Representative phase contrast images of macrophage morphology in different experimental conditions (**e**). Images were taken with IncuCyte using 20× magnification. Biomaterial particles are indicated with a white asterisk. All quantitative data are presented as the mean ± SD of one biological experiment with three technical replicates per each experimental condition and normalized to the first time point (0 h). p** ≤ 0.01 (ANOVA followed by Tukey's multiple comparisons test). LPS lipopolysaccharide

infection treatment and control along with its osteogenic effects. More recently, the focus has broadened on the possibility of using BG S53P4 as an immunomodulatory material. In the current study, the effects of BG S53P4 on macrophage activation, function, and immunometabolism were explored. According to the results from the current work, BG S53P4 increases both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of macrophages. In other words, BG S53P4 counteracts the suppressive effect of LPS on oxidative phosphorylation, but

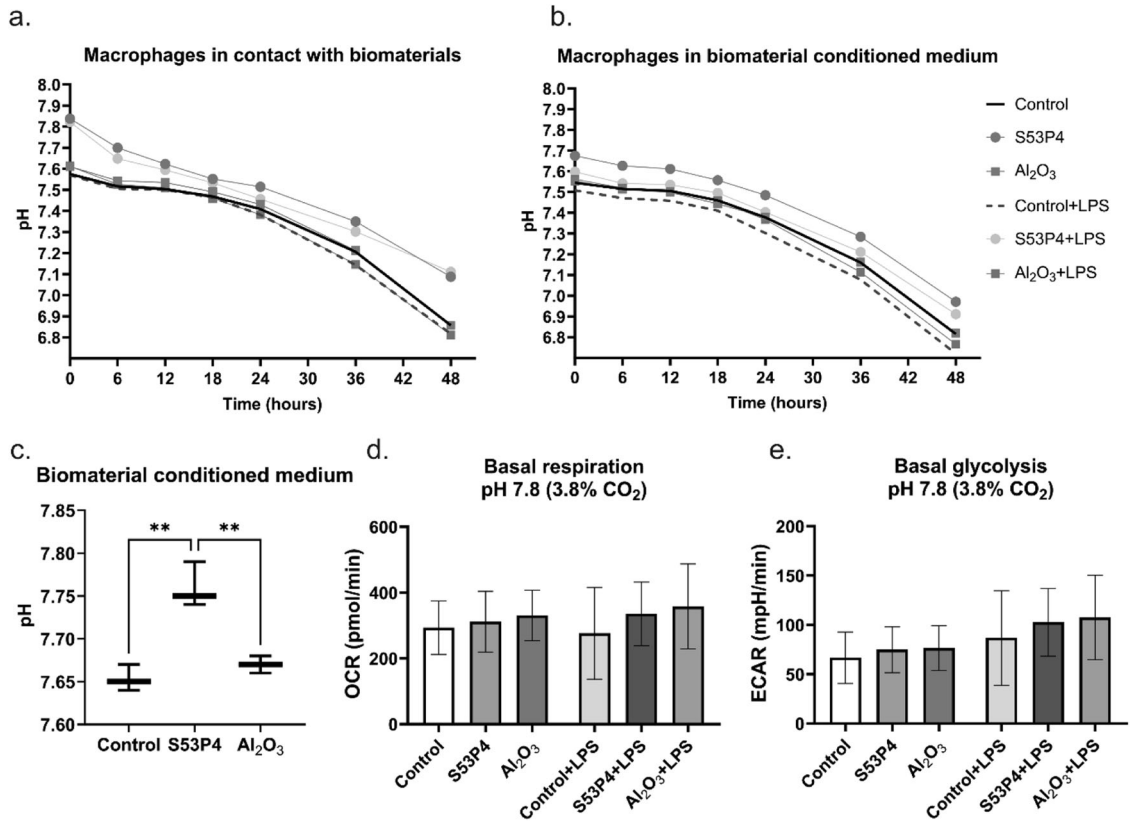


Fig. 5 The decrease of pH over time was measured in RAW 264.7 macrophage cultures (biological replicates n = 1) (**a**, **b**). The pH was also measured from cell culture medium without cells, which was conditioned with bioactive glass (BG) S53P4 for 48 h (experimental replicates n = 3) (**c**). The pH was adjusted to 7.8 by setting the CO_2 level in the cell culture incubator to 3.8% and the subsequent mitochondrial respiration rate of RAW 264.7 macrophages cultured in the presence of BG S53P4 or Al_2O_3 conditioned media was evaluated by the oxygen consumption rate (OCR) (**d**) and extracellular acidification rate (ECAR) (**e**) after 48 h (biological replicates n = 3, technical replicates n = 3 per group). All data are presented as the mean ± SD. p** ≤ 0.01 (ANOVA followed by Tukey's multiple comparisons test). LPS lipopolysaccharide

slightly increases the glycolysis induced by LPS. We also show that BG S53P4-exposed macrophages expressed lower mRNA levels of inflammatory cytokines *Il6* and *Il1b* in response to LPS. Furthermore, we observed lower activity of inflammatory IRF and NF-κB pathways, as well as decreased nitrogen oxide (NO) secretion. Thus, our results are in line with the consensus according to which bioactive glasses promote an anti-inflammatory phenotype of macrophages. Notably, our results were not dependent on direct contact between cells and BG S53P4, as demonstrated by the use of biomaterial-conditioned medium.

The mechanisms by which bioactive glasses affect macrophages are not fully understood. The use of conditioned medium in our experiments indicates that soluble factors are sufficient to cause a comprehensive change in macrophage energy demand and to downregulate several inflammation-related events, such as NO secretion. It has been recently shown by Sun et al. [16] that phosphate ions released from calcium phosphate ceramics can enhance the polarization of RAW 264.7 macrophages into anti-inflammatory cells via metabolic regulation [16]. The authors describe a significant increase in intracellular inorganic phosphate linked to elevated ATP production [16]. Their results are similar to the ones presented in the current study, as we also describe a highly energetic macrophage phenotype with not only an elevated mitochondrial respiration, but also with a higher glycolytic profile. Phosphate is one of the dissolution products of BG S53P4 [8], but further studies are needed to confirm its role in macrophage immunometabolism.

Regarding inflammatory phenotype, Barrak et al. [14] have shown that pro-inflammatory cytokines IL1 and IL6 are upregulated by titanium alloy in several clinically relevant cell populations, but the presence of S53P4 dissolution products suppressed this upregulation [14]. Additionally, the dissolution products of another bioactive glass, a more reactive 45S5, have been shown to reduce the *IL1b* mRNA expression in RAW 264.7 macrophages [17]. These observations demonstrate the potential of bioactive glasses as inflammation-modulating materials and highlight the importance of soluble factors. However, it is also important to note that in the current study, macrophages demonstrated

reduced mRNA levels of inflammatory cytokines *Il6* and *IL1b* in response to LPS after they had been cultured in direct contact with BG S53P4. In line with these results, a previous study has shown that pre-incubation of human macrophages in contact with BG S53P4 scaffolds during and prior to LPS activation results in decreased mRNA expression of *IL1b* and in decreased secretion of both pro-inflammatory cytokines IL1b and IL6 [13]. Thus, all this implicates that inflammatory cytokine profiles are similar whether macrophages are cultured in direct contact with BGs or in BG conditioned medium.

Certain factors related to macrophage function seem to be dependent on the applied cell culture-model, i.e., whether conditioned medium was used, or cells were grown in contact with the biomaterial. According to our results, BG S53P4 reduced the IRF pathway activation induced by LPS, but the reduction was more prominent when cells were cultured in direct contact with bioactive glass. BG S53P4 also reduced the NF-κB pathway activation induced by LPS, but conversely, the suppression was more pronounced when cells were cultured in BG S53P4 conditioned medium and not in direct contact with the BG. We also observed that macrophage cultures reached lower cell confluence when cultured in direct contact with biomaterials S53P4 or Al_2O_3. However, since both biomaterials had a similar effect, the likely explanation is the mechanical disturbance caused by material granules moving in the cell culture wells.

Given that BG S53P4 is known to increase the pH of its surroundings [8], it is tempting to speculate that this may be another mechanism by which BG S53P4 exerts its effects on macrophages. Massera et al. [18] have previously reported how the pH of cell culture medium containing BG S53P4 is first increased (pH 7.6), but over time decreased (pH 7.4) but after two weeks of incubation, no changes in the pH prior to and after medium change are observed [18]. The authors speculate that the reaction layer forming at the particle surface is slowing down the dissolution leading to pH stabilization. More recently, Björkenheim et al. [13] describe how BG S53P4 first increases the pH of cell culture medium from 7.4–7.8 in 1 h, after which the pH level slowly decreases to 7.6. They also reported higher pH levels in macrophage cell cultures with BG S53P4 [13]. In the current work, a difference of approximately 0.2 pH units between control and BG S53P4 conditioned medium was observed. Remarkably, this increase in pH caused by BG S53P4 was maintained in cell culture. We observed that still after 48 h, despite LPS activation, which generally leads to the acidification of cell culture environment, there were higher pH levels measured in macrophage cultures with BG S53P4 conditioned medium. As was already highlighted above in relation to macrophage metabolism, the efficacy of BG dissolution products appears impressive.

Nitric oxide (NO) is increasingly recognized as a regulator of macrophage phenotype and function, importantly in the context of immunometabolism [15]. In cells, enzymatically produced NO is oxidized into nitrite [19], which can then be measured to evaluate NO secretion. Low environmental pH has been shown to activate the inducible nitric oxide synthase NOS2 in macrophages leading to the accumulation of nitrite [20]. In the current work, the upregulation of NOS2 mRNA was detected 6 h after LPS activation. Remarkably, the upregulation was more pronounced in macrophages cultured in contact with BG S53P4 and was also detected in un-stimulated cells. Because BG S53P4, as discussed above, leads to the alkalization and not acidification of medium, there may be another factor apart from pH that causes the initial NOS2 transcription related to BG S53P4.

Despite the initial increase in NOS2 mRNA, the measured levels of the final product, i.e., NO, were actually lower in LPS-stimulated macrophages with BG S53P4 both in contact and in conditioned medium compared to LPS-activated control cells. In a healthy state, NO production from macrophages is low and increased levels are achieved during inflammation [15]. Therefore, in this regard, macrophages cultured with BG S53P4 resemble the non-inflammatory cells. However, it should be noted that NO flux in cells is affected by many variables, including the density of cells expressing NOS2 [21]. In the current study, NO levels were only measured 24 h after LPS stimulation and therefore the increase could actually have happened prior to this time point in macrophages predisposed to BG S53P4.

Tumor necrosis factor (TNF) is an effective facilitator of inflammatory responses but the reports concerning the effects of bioactive glasses on TNF expression are conflicting. Our results from the current study show that BG S53P4 alone increased the mRNA expression of *Tnf* in un-stimulated RAW 264.7 macrophages. These results are in line with previous research by Björkenheim et al. reporting that BG S53P4 increases both the mRNA level and secretion of TNF in human macrophages [13]. However, Björkenheim et al. discovered that when macrophages were cultured in contact with BG S53P4 and then stimulated with LPS, the previously increased TNF mRNA and protein levels decreased [13]. In the current study, LPS stimulation of macrophages after BG S53P4 contact had however no major effect on *Tnf* mRNA levels. The conflicting reports are not limited only to BG S53P4 as another BG 45S5 has been shown to increase the secretion of TNF in mouse peritoneal macrophages [22], while others have reported lower TNF levels in the presence of BG 45S5 [23]. Even though the differences could be partly explained by the diverse experimental designs with different cell types and species, and whether the analysis was performed on mRNA or protein level, the effects of LPS described above should not be overlooked, as they demonstrate how different BGs

might result in different responses when macrophages are exposed to inflammation-inducing factors.

Our results support the current understanding of the multidimensional nature of macrophage polarization and limitations of the dualistic view of macrophages as either inflammatory (M1) or anti-inflammatory (M2). For example, as discussed above, BG S53P4 increased *Tnf* mRNA expression, while the mRNA levels of *Il1b* and *Il6* were downregulated. TNF is a known anti-M2 polarizing factor [24], suppressing *Arg1* and other M2-macrophage related genes [25]. It is thus possible that in our experimental setting, the BG S53P4 guides the macrophages towards anti-inflammatory functions as evidenced by the low *Il1b* and *Il6* mRNA expression. However, at the same time, macrophages were exposed to LPS mimicking the presence of bacteria, which could result in TNF expression hindering macrophage polarization towards anti-inflammatory M2-phenotype until the microbial products are cleared. According to this hypothesis, BG S53P4 might also modulate the expression of certain interleukins and TNF via different pathways.

According to the results presented here, BG S53P4 regulates the phenotype of macrophages in a more discreet manner, than simply causing a complete reversal of phenotype, e.g. from inflammatory M1 macrophages to anti-inflammatory M2 macrophages. The addition of IL4, which is used to produce typical M2(a)-polarized macrophages [26], could have induced a clearer phenotype in the presence of LPS and BG S53P4. However, as such defined polarization was not in our focus, this was not addressed in the current study. Moreover, the functional analysis of macrophages was done on reporter activity, mRNA, and enzyme activity levels. The protein expression levels may differ from these data, which needs to be considered when interpreting the results presented here. Future analysis of protein levels in this context would provide additional insights into macrophage responses to BG S53P4.

BG S53P4 has shown great efficacy in the treatment of chronic osteomyelitis and septic non-union fractures [9, 27]. While systemic antibiotics are still required in the treatment of these conditions, one of the major advantages of BG S53P4 is the antimicrobial nature of the material, which results in a similar infection clearance at the surgical site compared to other biomaterials loaded with antibiotics [28]. In other situations, such as in diabetic wound healing, the local antimicrobial effects of BG might be sufficient to reduce the need for antibiotics. Macrophages isolated from chronic wounds in diabetic patients have demonstrated a proinflammatory phenotype, which may be further exacerbated by the wound environment [29], and there is also a pathologically high production and secretion of inflammatory cytokines by macrophages in diabetic wounds [30]. Given that our findings demonstrate that BG S53P4 downregulates *Il1b* mRNA expression in macrophages, an observation supported by reduced cytokine secretion as shown by others [13], and considering that blocking IL1b activity is known to downregulate the proinflammatory macrophage phenotype in chronic wounds [29], therapies utilizing BG S53P4 could be particularly advantageous for diabetic wound healing.

5 Conclusions

In the current study, we demonstrate how BG S53P4 reverses the suppression of oxidative phosphorylation in LPS-activated macrophages and guides the cells towards an anti-inflammatory phenotype, characterized by reduced *Il1b* and *Il6* mRNA expression, lower inflammatory pathway activation and decreased NO secretion. These changes did not depend on macrophages being in direct contact with BG S53P4 indicating an important role for indirect effects, such as the dissolution products or the pH increase associated with BG S53P4. These indirect effects of BG S53P4 probably play a significant role in modulating the inflammatory phenotype in macrophages; however, metabolites from aerobic glycolysis are also known to regulate immune responses [31]. While the bioactive glass discussed in this study is already in clinical use, our novel findings on the metabolic mechanisms by which BG S53P4 modulates macrophage immunomodulatory properties offer valuable insights for refining its clinical applications. However, a critical perspective emphasizes the need for further study into how these mechanisms may translate into improved patient outcomes. This work nevertheless lays the groundwork for the therapeutic potential of bioactive glasses in managing conditions where inflammation is prominent, such as wound healing.

Data availability

The data that support the findings of this study are available from the corresponding author (Jorma Määttä, jmaatta@utu.fi) upon reasonable request.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s10856-025-06861-y.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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